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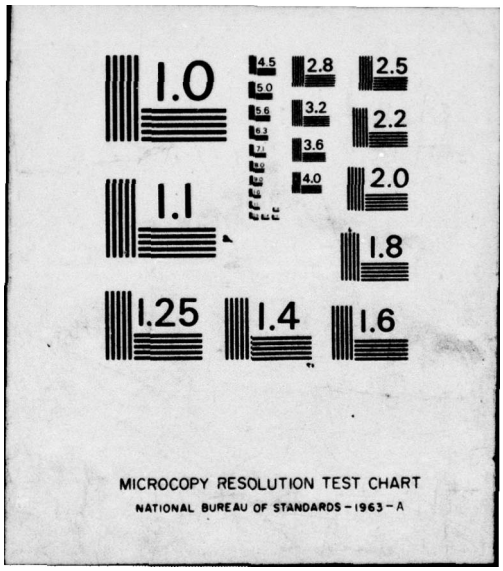
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Immunity to Malaria Parasites

Final Technical Report

R.S. Phillips

Department of Zoology

University of Glasgow

Report Date - July 1976

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AB

20. Plasmodium falciparum

A standard procedure for the growth and multiplication of P. falciparum in human blood in microtissue culture trays is described.

Out of over 100 Gambian and 80 Nigerian sera tested for their ability to inhibit reinvasion of P. falciparum in vitro, relatively few were strongly inhibitory. Some sera were inhibitory to parasites from some donors but not from others. The heterogeneous nature of the P. falciparum populations could account for this. It was found to be possible to circumvent this problem and relate immune sera to the specific population of P. falciparum against which they were raised by cryopreserving the parasite while the patient was treated and recovered from his infection. P. falciparum, cryopreserved at the ring stage subsequently resume growth and reinvade in culture. Post-treatment sera had strong homologous and some heterologous inhibitory activity in vitro. The great potential of the use of cryopreserved P. falciparum in immunological and biochemical studies is emphasised.

Murine malaria

Attempts to demonstrate further the antigenic lability of P. chabaudi by inducing immune serum resistant populations were negative. The possible role of regulatory mechanisms in the immune response contributing to the survival of malaria parasites was discussed.

The individual and cooperative roles of T- and B-cells in immunity to P. chabaudi are discussed.

Contents

Summary:

Plasmodium falciparum

A standard procedure for the growth and multiplication of P. falciparum in human blood in microtissue culture trays is described. Comparable multiplication rates were obtained when the parasite was introduced into the microcultures at the ring or trophozoite and schizont stages. Addition of fresh red cells just before reinvasion did not significantly improve the multiplication rates.

Incorporation of ^{3}H-isoleucine into P. falciparum after reinvasion was shown to be a sensitive measure of the reinvasion rate. Lactate production was also shown to be a useful indicator of the parasite's growth in vitro.

Out of over 100 Gambian and 80 Nigerian sera tested for their ability to inhibit reinvasion of P. falciparum in vitro, relatively few were strongly inhibitory. Some sera were inhibitory to parasites from some donors but not from others. The heterogeneous nature of the P. falciparum populations could account for this. It was found to be possible to circumvent this problem and relate immune sera to the specific population of P. falciparum against which they were raised by cryopreserving the parasite while the patient was treated and recovered from his infection. P. falciparum, cryopreserved at the ring stage subsequently resumes growth and reinvades in culture. Post-treatment sera had strong homologous and some heterologous inhibitory activity in vitro. The great potential of the use of cryopreserved P. falciparum in immunological and biochemical studies is emphasised.

Murine malaria

Attempts to demonstrate further the antigenic lability of P. chabaudi by inducing immune serum resistant populations were negative. The possible role of regulatory mechanisms in the immune response contributing to the survival of malaria parasites is discussed.

It was confirmed that BCG given intravenously was a powerful potentiator of the immunity induced by irradiated P. berghei. Heated P. chabaudi were less immunogenic than irradiated parasites. Irradiated rings, trophozoites and schizonts of P. chabaudi were equally immunogenic.

P. berghei depressed the ability of mice to express a primary and a secondary immune response to Trichinella spiralis and Babesia hylomysci and B. microti infected mice had impaired ability to expel a primary Trichuris muris infection.

Spleen cells from mice immune to P. chabaudi were fractionated on nylon wool columns and adoptively transferred to 600 rad or 800 rad irradiated recipients. Unfractionated spleen cells and enriched T- and enriched B-cell populations all protected to some degree but the latter two populations invariably less than the unfractionated cells in 800 rad recipients.

The individual and cooperative roles of T- and B-cells in immunity to P. chabaudi are discussed.

Irradiated mice were again found to have initially some non-specific resistance to P. chabaudi which potentiated the activity of immune serum.

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The work described in this report was carried out with the help of Research Assistant, Mr V. McDonald, and with the technical help for part of the time, of Mrs C.D. Pearson.

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INTRODUCTION

Malaria is still a major health problem in many parts of the world. Nearly 500 million people are living in areas where there are few or no control measures against the disease (W.H.O., 1973). It is likely that the control and eradication measures which have hitherto proved effective in ridding some parts of the world of the disease are not going to be so successful in these remaining affected areas. Africa presents the most difficult problem. Undoubtedly the existing control measures will have to be supplemented by alternatives and the development of an antimalarial vaccine would represent a major step forward. The production of this vaccine will ultimately be dependent on the development of techniques which will permit the unlimited growth in vitro of the relevant stages in the life cycle of the malaria parasite. Such techniques are not yet available.

It is becoming clear that the immune response to an antigen is complex and that the type of immune response which the antigen evokes is the end result of the interaction of a number of regulatory systems. In experimental situations at least it now appears possible to manipulate to some extent these regulatory mechanisms in order to promote one rather than another aspect of the immune response to an antigen. Malarial antigens, either from living or dead parasites, when presented in the form of a vaccine will, it is hoped, provoke those immune responses which will give protective immunity against the parasite. It may, however, be necessary or expedient, in order to induce an effective immunity by vaccination, to potentiate by manipulation of the regulatory mechanisms those aspects of the immune response to malaria parasites which are associated with antimalarial protective immunity. A detailed knowledge of the character of the immune responses to malaria parasites and of the ways in which immunity acts against the parasite will indicate in which areas potentiation of the immune responses to malarial antigens will be beneficial. It was principally for this reason that the studies described in the report have been pursued.

Investigations have been carried out in the following areas:

(a) mechanisms of protective malarial immunity with especial regard to the lymphoid cells involved;

(b) survival of malaria parasites in the immunized host;

(c) adjuvants as effective potentiators of protective immunity when used in conjunction with irradiated parasitized cells as a vaccine;

(d) acute protozoal infections and the host's immune response to other parasites.

This study conveniently falls into two parts. The first is concerned with immunity to the human malaria parasite, Plasmodium falciparum, and the second with immunity to and immunosuppression by the rodent malarias in the mouse. The report will relate the findings and conclusions from each part separately and then draw them together in a concluding section.

PART I

IMMUNITY TO THE HUMAN MALARIA PARASITE

PLASMODIUM FALCIPARUM

Introduction

This part of the study was carried out in cooperation with Dr R.J.M. Wilson who is on the research staff of the Medical Research Council (M.R.C.) at the National Institute for Medical Research, Mill Hill, London. The work was carried out at the M.R.C.'s Laboratories at Fajara, The Gambia, in West Africa, through the kind permission of the Director, Dr R.S. Bray, and with the help of the resident technical and medical staff.

The Gambia is a hyperendemic malarious area. P. falciparum accounts for almost all the cases of malaria with the occasional case of P. ovale and P. malariae. Although malaria transmission occurs throughout the year in The Gambia, in most parts the majority of cases occur during, and for two or three months after, the annual rainy season which usually lasts from July to October. There is a high mortality rate from the disease in children between 3 months and 3-4 years old. Children who survive these early years gradually build up an effective immunity. Up to adolescence this immunity, an antitoxic immunity, frequently is only sufficient to prevent children showing clinical symptoms in spite of carrying detectable parasitaemias but by adulthood a strong immunity has been established. Even at this stage in their development occasional parasitaemic episodes can be detected in the Gambians. The important point is that even in individuals living all their lives within one locality it takes several years of exposure to P. falciparum before an effective immunity to the parasite is attained and even in immune individuals the parasite may persist albeit at a chronic and usually sub-patent level. A number of possible explanations can be proposed to explain why the establishment of a beneficial level of acquired resistance takes so long and even when this has been achieved why parasites may still survive and multiply. Two possibilities will be discussed at this stage. First, it is likely that even within one locality there exists a large number of 'strains' of P. falciparum, each of which necessitates a specific immune response from the host and that it is several years before any one individual has been exposed to most of the existing strains within a locality. It might, however, be anticipated that sensitization to one 'strain' would promote a more rapid response to others. Secondly, during the course of the asexual blood phase of an infection following the bite of a single mosquito, the parasite may be able to change repeatedly its antigenic character in order to escape from the effects of the host's immune response against it, i.e. the parasite might undergo the pattern of antigenic variation so characteristic of the sleeping sickness trypanosomes and spirochaetes. It is quite clear that in the preparation of an antimalarial vaccine it will be very important to establish whether P. falciparum does exhibit the degree of antigenic variability suggested above. The study carried out in The Gambia and reported here was designed to try and give information in this area.

Immunity to P. falciparum in man, in part at least, is humoral. Cohen et al. (1961) pooled serum from a number of adult Gambians, separated out the IgG fraction and showed that this IgG had therapeutic activity when injected into children with acute P. falciparum infections. The immune IgG appeared to act when the parasite in red cells reached the mature (schizont) stage. Such passive transfer studies for practical reasons, however, could clearly not be readily extended to examine the effect of serum from single individuals.

The asexual blood phase of the monkey malaria, P. knowlesi, can be grown through a complete cycle in vitro (see Trigg, 1969). Multiplication rates of 5-8 times can be obtained when cultures are initiated with late trophozoites or early schizonts. Multiplication rate is usually defined in these studies as follows:

$$\text{Multiplication rate} = \frac{\text{no. of parasites per } 10^4 \text{ red cells after } (0 + x) \text{ hrs}}{\text{no. of parasites per } 10^4 \text{ red cells at 0 hrs}}$$

During the interval between 0 hrs and (0 + x) hrs, the parasite will have passed through one schizogony and the released mesozoites invaded more red cells. Brown et al. (1968, 1970) and later Cohen & Butcher (1970) used this culture system to show that the serum from monkeys immune to P. knowlesi can reduce the rate of reinvasion of fresh red cells and hence the multiplication rate of P. knowlesi in vitro. The growth of P. knowlesi from ring to schizont stages within the red cell was not affected by the immune serum. This culture system therefore provided a sensitive measure of the antiparasitic activity of at least one type of antibody in the serum of immune monkeys. Phillips et al. (1972) extended these studies on simian malaria to P. falciparum in man. These workers devised an in vitro sub-culture technique for the asexual blood stage of P. falciparum. Infected blood was taken from infant Gambians and the asexual blood stage was grown through two and a half cycles. Multiplication rates of 3-8 times were achieved after reinvasion in the first cycle of development. It should be noted that the in vitro culture of P. falciparum poses two major problems not associated with P. knowlesi. First, the former parasite takes 48 hrs to complete its asexual growth cycles in the blood compared with 24 hrs for P. knowlesi and secondly, P. falciparum but not P. knowlesi withdraws from the peripheral circulation at the late ring stage. This necessitates that P. falciparum infected blood is introduced into in vitro culture at a relatively early stage in the parasite's asexual growth cycle. Phillips et al. (1972) subsequently examined the ability of fifteen sera taken from adult Gambians to inhibit reinvasion in vitro by P. falciparum and of these sera two had clear inhibitory activity. This then was the position when the first U.S. Army supported study was undertaken. This study was described in the Annual Report (Contract DAJA-37-73C-3492).

The important points from that report can be summarised here before describing the subsequent investigations.

The subculture technique used by Phillips et al. was carried out on a relatively large scale and the amount of each serum sample used in each test for inhibition of reinvasion was correspondingly large. Only a few tests could be carried out with each serum sample. Thus the in vitro culture of the blood stages of P. falciparum, still using a subculture technique, was reduced to a microscale, the parasites being grown in conventional 96 well microtissue culture plates. The procedure had many features in common with that of Diggs et al. (1971). Using this microculture technique populations of P. falciparum from different infants were grown through one cycle of schizogony and reinvasion in the presence of serum from

60 Gambians from 11 to 60 years old. The majority of the sera had some but relatively small inhibitory activity on the parasite's ability to invade new red cells. Of the markedly active sera some were active against populations of P. falciparum from some children but not others suggesting that there may be some antigenic diversity amongst the populations of P. falciparum in that part of the Gambia.

The present report is concerned with the following areas of investigation which followed on from our first study.

1. Modification and simplification of in vitro microculture of P. falciparum infected human blood.

2. Use of radiotracers for monitoring growth of P. falciparum in microcultures. Examination of the possibility of measuring lactate production as a measure of the parasite's growth in vitro.

3. Growth of P. falciparum in vitro after removal of white cells from infected blood.

4. Examination of further Gambian sera and a few Nigerian sera for inhibitory activity.

5. In vitro culture of cryopreserved P. falciparum in the presence of pre- and post-treatment serum from the donor of the parasitized blood.

The results from each of the five investigations will be considered separately and then together in a concluding statement.

1. Modified in vitro microculture technique for P. falciparum

The asexual blood stage of P. falciparum has a 48-hr growth cycle. Young ring stages are usually first detected in the peripheral blood between 0500 and 0800 hrs. The rings grow bigger over the next 24 hrs and just before they become early trophozoites, between 0800 and 1200 hrs on the second day, they withdraw from the peripheral circulation. The parasite reappears in the peripheral circulation the following morning when schizogony has been completed and the next brood of young rings has been produced. Ideally, infected blood for culture purposes is taken from infants by venepuncture when the parasites are at late ring stage but younger rings can be successfully grown. Heparin at final concentration of 17 i.u./ml is the anticoagulant. Children carrying a single brood of parasites and a parasitaemia of 2-5% are usually selected. Best multiplication rates are usually obtained with low parasitaemias. Higher parasitaemias can be cultured and if necessary the blood can be diluted with washed normal (Caucasian) red cells. The parasites are grown in tissue culture medium 199 supplemented with sodium bicarbonate and glucose to give final concentrations of 0.198 g/l and 3 g/l respectively; penicillin at 200 units/ml, streptomycin at 100 units/ml and foetal calf serum to a final concentration of 5% are also added. Cultures are incubated at 37°C in a gas phase of 95% air, 5% CO_2 to give a final pH of 7.4. Alternatively, the parasites can be grown as effectively in supplement TC 199 additionally buffered with 20 mM Hepes.

Table 1

Comparison of multiplication rates after the same sample of infected blood was either put straight into microtissue culture trays of after subculture

Procedure	Volume of infected blood µl	Multiplication rates for three blood samples 7545	7546	7549
Straight into tray	5	2.9	2.3	2.3
	10	3.6	2.4	2.6
	15	2.6	2.0	3.0
Subculture into tray	5	2.7	2.1	1.9
	10	3.0	2.8	2.2
	15	3.0	2.4	2.1

Table 2

Predominant stages present during culture of blood from child 7545

Stage	Time		
	Day 1	Day 2	
	2130 hrs	0930 hrs	2130 hrs
Young ring		+	+
Medium ring	+++		+
Large ring		+++	
Young trophozoite			+++
Late trophozoite			
Schizont	+	+	

Each microculture contains a total volume of 260 µl consisting of 10 µl infected blood in serum (see below), up to 100 µl human serum and the rest supplemented TC 199. The tissue culture trays were kept stationary. The microculture of P. falciparum are set up in one of two ways, either by introducing the parasites into the tray at the ring stage or after subculture at the late trophozoite or schizont stage. In the first case the infected blood is washed twice with TC 199 buffered with Hepes and resuspended to the original blood volume (haemocrit 40%) with Caucasian AB serum (occasionally but satisfactorily with autologous serum) before being added to the microcultures. In the second case the infected blood is grown to trophozoite or early schizont stage in bulk culture on a shaking water-bath (Phillips et al., 1972), washed twice with Hepes buffered 199 or modified ringer (Geiman et al., 1966) resuspended to the original blood volume in Caucasian AB serum (occasionally in autologous serum) and then added to the microcultures. It was found that heparinized whole blood added directly to the microcultures occasionally clotted over the period of the culture and that by washing the infected blood and resuspending the red cells in AB serum this problem was overcome. The multiplication rates obtained following the two procedures were usually comparable as illustrated in the following experiment. Infected blood from three children, designated 7545, 7546 and 7549, was grown in microculture by both procedures. Microcultures were set up containing 5, 10 or 15 µl of infected red cells resuspended in AB serum, 50 µl AB serum and 200 µl supplemented TC 199. All three children were bled at 1130 hrs (day 0) and the starting parasitaemias ranged from 3.6 to 5.7%. 7545 consisted of predominantly large rings with a minor population of small rings, 7546 mainly of medium rings and a few small rings and immature gametocytes, and 7549 again mainly of medium rings with minor populations of smaller and larger rings. Subculture of 7545 was carried out at 0020 hrs on day 1 when the major population of parasites was at the late trophozoite stage. 7549 and 7546 were subcultured at 0830 hrs on day 1 when the major population of parasites was late rings. The parasites were cultured in the trays for five days and at intervals, the red cells from 2 or 3 wells were smeared, Giemsa's stained, and examined. The multiplication rates for each sample of infected blood was determined after the major parasite population has gone through schizogony and had reinvaded, and these are given in Table 1 both for the infected blood samples introduced straight into the trays and the subcultured blood. It can be seen that for each sample of infected blood the multiplication rates are broadly similar irrespective of the method of culture or whether 5, 10 or 15 µl of resuspended red cells were put into each microculture at the start.

This experiment also demonstrated that where more than one brood of parasites is present and within each brood the parasites are growing synchronously in the donor blood both broods are able to grow in the microcultures and go through schizogony. For example, the predominate stage of parasites present in 7545 on days 1 and 2 of the culture is shown in Table 2. By 2130 hrs on day 1 the major population of parasites had already gone through schizogony, reinvaded and the new brood of rings made some growth. The minor population, initially small rings, was about 24 hrs behind the major population and it went through schizogony on day 2. Similarly, it was possible to follow the growth of the major and minor populations of parasites in 7546 and 7549. It is therefore possible when examining, for example, the inhibitory activity of immune sera, if the cultures are initiated with infected blood containing two broods of P. falciparum, the inhibitory activity of the immune sera on each of two broods can be determined. Where more than one brood of parasites is present it is, of course, likely that they have arisen from sporozoites from different mosquitoes, and may represent antigenically different populations of P. falciparum.

Table 3

Effect of replacing some of the medium in the microculture

Blood sample	Medium replaced	Mean* % parasitaemia		
		Day 3	4	5
7545	+	8.2	6.4	7.4
	-	11.2	7.9	5.2
7546	+	14.3	14.4	7.4
	-	16.3	11.4	8.9
7549	+	16.2	11.2	8.0
	-	10.9	9.6	N.D.

N.D. - not done

*Mean of 2 or 3 cultures

Table 4

Addition of fresh red cells to microcultures of P. falciparum

	Infected blood sample	Time fresh red cells added				
		Day 1				Day 2
		0120 hrs	1100 hrs	1545 hrs	2130 hrs	0930 hrs
Multiplication rate	7545	3.3	1.9	2.4		
	7546	2.8		1.6	1.3	1.1

Multiplication rate without additional red cells for 7545 x 2.9 and 7546 - x2.3

The major brood of parasites in 7545 went through schizogony between 1200 and 2000 hrs on day 1 and in 7546 between 0000 hrs and 0800 hrs on day 2

It is usually more convenient to set up microcultures with the infected blood directly from the donor but there are occasions when it is desirable or more convenient to grow the parasites in a bulk culture for a period and later carry out a subculture.

Two other procedures were examined to see if they improved the growth and multiplication rate. First we tried replacing some of the culture medium but no clear benefit was gained from this. For example, in the experiment described above in some of the microcultures of blood from donors 7545, 7546 and 7549 set up with 10 µl of resuspended blood cells per culture, 100 µl of the culture fluid was replaced with an equal volume of fresh supplemented 199 on day 2 and the red cells resuspended. The parasitaemias on days 3, 4 and 5 in these cultures and in similar ones in which the medium was no replaced are given in Table 3. It can be seen that there is little difference and the morphological appearance of the parasites in the stained-blood smears suggested that changing some of the medium had not improved parasite viability. It is emphasised that one of the problems of assessing parasite numbers of P. falciparum by examination of stained blood smears is that trophozoites and schizonts especially tend to be congregated in the tail of the blood smear which inevitably leads to a high degree of error in counts from these smears.

The second procedure we tried for improving multiplication rates was to add fresh red cells to the cultures when the parasite had reached a trophozoite or schizont stage. Our experiments can only be considered preliminary at this stage but the results do not suggest that any significant benefit is gained. The red cells to be added were taken from RSP, washed 3 x times with modified Ringer and resuspended in AB serum to the original blood volume. They were stored at 4°C. A representative result is described. Microcultures were set up each containing 200 µl TC199, 50 µl AB serum and 5 µl of infected blood 7545 or 7546 as used in the experiments described above (not subcultured). 10 µl of washed resuspended RSP cells were added to some of the wells, and the contents of each well mixed at the times shown in Table 4. When the major population of parasites had gone through schizogony and reinvaded smears were made: for 7545 this was on day 2 at 2130 hrs and for 7546 on day 3 at 0930 hrs. The multiplication rates were then calculated taking into account the dilution factor introduced by adding the additional red cells. In other experiments fresh red cells were added at subculture when the parasites were at the trophozoite stages and in a proportion of these experiments the multiplication rate appeared to be slightly enhanced. Remaining preliminary experiments of this type compared the multiplication rates after additional RSP red cells were added to microculture at the trophozoite stage when the extra red cells had been maintained beforehand at 4°C or 37°C for up to 12 hrs. It was thought that red cells which had been metabolically active in vitro at 37°C for a period of hours might be less able to receive and to support the growth of P. falciparum, than similar red cells previously kept at 4°C. These experiments were of a preliminary nature but the results suggested that where the red cells had been maintained at 37°C slightly lower parasitaemias were attained after reinvasion had taken place.

A feature of our microcultures of P. falciparum is that after the parasite has gone through schizogony and reinvaded a proportion of the newly infected red cells are multiparasitized and may contain up to 6, rarely more, ring stages. Few of those multiparasitized red cells containing more than 2 rings are able to support growth to maturity of all the parasites they contain. Red cells containing up to

Table 5

Survival of multiparasitized red cells in microcultures

Stage of parasite	No. of parasites per red cell			
	1	2	3	4
Ring	300(72)	88(21)	19(5)	8(2)
Trophozoite	238(76)	61(19)	11(3)	5(2)
Schizont	290(95)	14(5)	1(<<1)	0

Figures in ( ) are the numbers in each category expressed as a percentage of the total parasitized red cells counted.

4 small trophozoites have frequently been observed, often some trophozoites appear to be growing at the expense of others within the same red cell but beyond the trophozoite stage those red cells containing more than 2 parasites are either lost from the culture or the parasites in excess of 2 are lost from the red cell. For example, smears were made from microcultures shortly after reinvasion when rings were present and on later occasions when trophozoites and eventually schizonts were present. Separating the parasitized red cells on each occasion according to the number of parasites they contain gives the results in Table 5. It can be seen that the number of red cells containing 2 or more parasites is reduced from 24-28% to 5% by the time the parasites reached the schizont stage.

2a. Use of a radiotracer in microcultures of P. falciparum

Hitherto assessment of the growth of P. falciparum in the microcultures was by morphological examination of Giemsa's stained blood smears. This is not a technique suitable for detecting small differences between cultures. We have, therefore, in some experiments measured the uptake of H^3-isoleucine into protein by the parasite. The standard procedure was to add 1 µCi H^3-isoleucine (specific activity 26 Ci/m mol) in 10 µl of TC 199 to each 260 µl culture: the tracer was added at the start of the culture, during or after schizogony depending on the requirements of the particular experiment. At the end of the culture or at suitable time intervals the radiolabelled parasitized or control red cells were prepared for liquid scintillation counting as follows. The blood cells from each culture were deposited by centrifugation, carefully washed three times with modified Ringer, transferred with a minimum volume of Ringer to scintillation vials, mixed with two drops of hydrogen peroxide (30 v/v) and then left overnight at room temperature or heated to 50°C for one hour. Finally the bleached cells were dissolved in 0.5 ml of the tissue solublizer NCS, 5 ml of scintillation fluid was added and the vials were counted in a Packard liquid scintillation counter. In spite of the number of washes the blood cells were subjected to, the counts obtained for similar cultures were very close.

To illustrate the use of ^{3}H-isoleucine in the microcultures an experiment is described in which the tracer was added to the parasites either before or after schizogony. The reason for carrying out the experiment was as follows. A reflection of the amount of reinvasion by the parasites after schizogony would be the amount of incorporation of the tracer by the new generation of P. falciparum in the period between reinvasion and the end of the experiment. If the tracer is added to the microcultures before reinvasion, when the parasites at the late trophozoite or schizont stage, there will be some incorporation before schizogony and if this is appreciable it could hide any apparent differences in the uptake of label by the parasites after reinvasion which results from differences in the amount of reinvasion. Infected blood (5.8%) was taken at 1030 hrs on day 0 and set up in bulk culture. At 1200 on day 2, the parasites were washed, resuspended in AB serum and sub-cultured into microcultures as follows:

Table 6

Incorporation of ^{3}H-isoleucine into P. falciparum in microtissue cultures

	C.p.m. x 10^{-3}		
Time culture terminated	Day 2	Day 3	Day 4
	1330 hrs	0945 hr / 2345 hr	2315 hr
Row A	0.8	10.8(1) 19.7(3)	57.4(5)
		10.6(2) 18.8(4)	70.0(6)
C	0.6	9.7(1) 23.7(3)	64.2(5)
		11.0(2) 23.9(4)	75.8(6)
G	0.1	0.1(1) 0.2(3)	0.1(5)
		0.1(2) 0.1(4)	0.1(6)
A		5.5(7) 15.6(9)	67.3(11)
		4.8(8) 14.3(10)	60.4(12)
C		6.1(7) 17.9(9)	48.9(11)
		5.9(8) 18.4(10)	53.5(12)
B	0.1	0.7(1) 1.0(3)	4.2(5)
		0.7(2) 1.3(4)	2.8(6)
D	0.1	0.6(1) 1.9(3)	3.0(5)
		0.6(2) 1.7(4)	3.5(6)
H	0.04	0.03(1) 0.07(3)	0.04(5)
		0.02(2) 0.04(4)	0.04(6)
B		0.6(7) 1.9(9)	6.8(11)
		0.6(8) 2.0(10)	5.8(12)
D		0.7(7) 1.7(9)	5.6(11)
		0.6(8) 1.7(10)	6.4(12)

In ( ) well number

Parasitaemias of culture from stained blood film

	Ring	Trophozoite	Schizont	Rbcs	P/100 rbcs
Day 2 - 1330 hr	2	2	23	490	5.5
" 1800 hr	27	1	9	514	7.2
Day 3 - 0945 hr	all rings				13.7
" 2200 hr	all rings	-			12.4
Day 4 - 0900 hr	17	13	0	317	9.5

Row		
A	1μCi/well	10 μl infected blood/well
B	0.1μCi/well	
C	1μCi/well	5 μl infected blood/well
D	0.1μCi/well	
G	1μCi/well	10 μl non-infected blood/well
H	0.1μCi/well	

Each well (12 per row) contained 200 μl medium + 50 μl AB serum. To wells 1-6 inclusive of all rows ^{3}H-isoleucine (specific activity 26 Ci/m mol) was added as indicated on day 2 at 1330 hrs when the parasites were almost all schizonts. To wells 7-12 inclusive of rows A, B and D, tracer was added on day 3 at 0030 hrs. Schizogony occurred after 1330 hrs on day 2 and was essentially completed by 0030 hr on day 3. Duplicate cultures were processed for tracer incorporation at 0, 20.25, 34.25 and 57.75 hrs after the microcultures were started.

The level of tracer incorporation is given in Table 6. It can be seen that in cultures of non-infected blood there was no significant incorporation of the tracer. Tracer added at the rate of 1 μCi per microculture gave a significant level of incorporation: 0.1 μCi was too small a dose. The amount of incorporation was approximately the same for the cultures initiated with 5 μl and 10 μl. From observations of stained blood smears in other experiments we have found that the multiplication rate in cultures initiated with 5 or 10 μl of infected blood was about the same. The results of these incorporation experiments suggest that with the starting parasitaemia of 5.7%, that 5 μl per well (final dilution of 1 in 50) might give better growth. Comparison of the incorporation rates when the tracer was added before or after schizogony, shows that the level of incorporation after 20.75 and 34.25 hrs of culture was higher when the tracer was added before schizogony. After 57.57 hrs, in the 10 μl cultures the mean incorporation was the same for the two times of tracer addition but in the 5 μl cultures it was still slightly higher in those cultures in which the tracer was added before schizogony. It is also noteworthy that there was a good degree of consistency in the counts for the members of each pair of cultures.

The results therefore showed that there was incorporation of ^{3}H-isoleucine throughout the culture period, both before and after schizogony. It appeared that where incorporation of the tracer was being used as a measure of reinvasion, it would be more satisfactory to add the tracer after reinvasion had occurred.

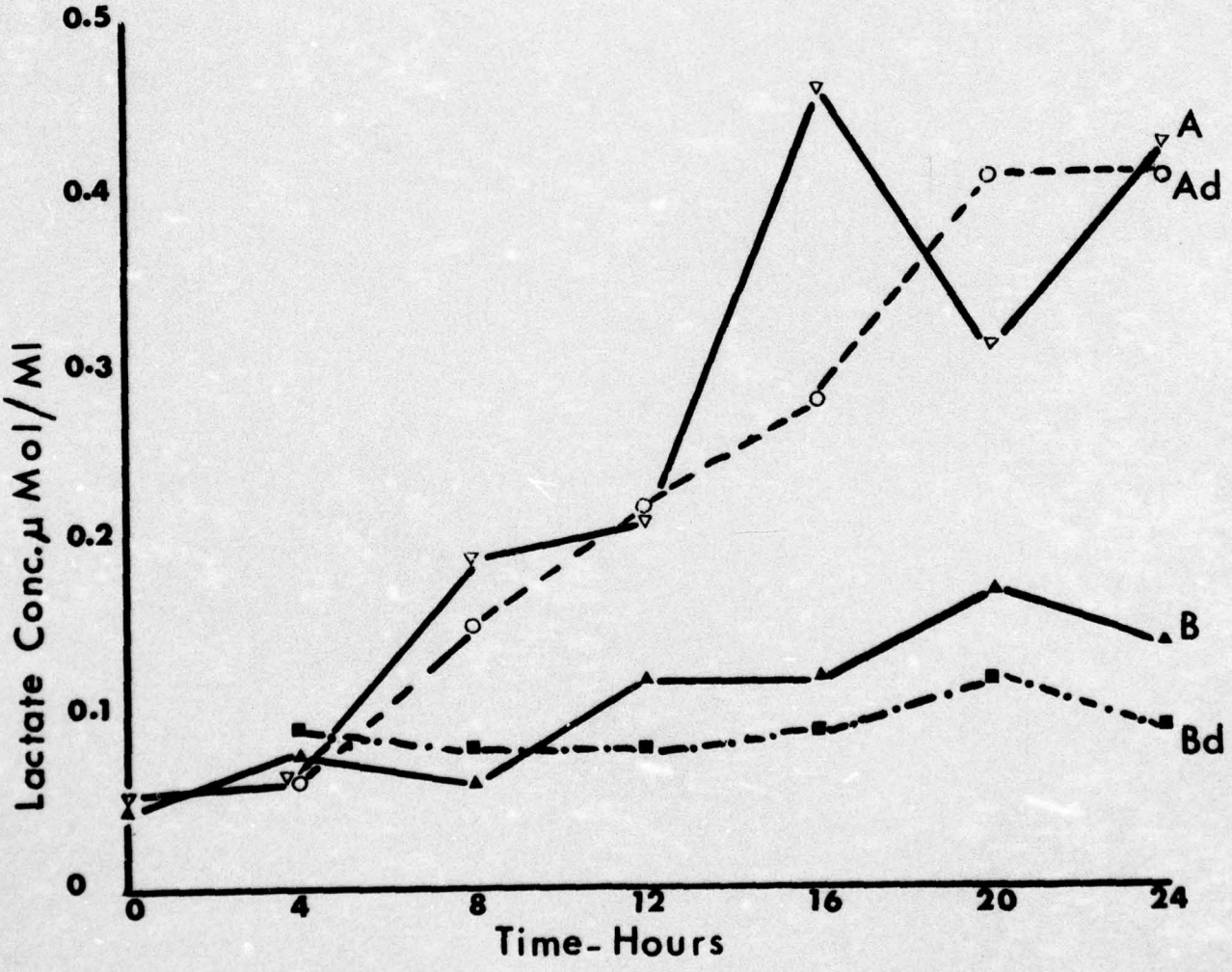


Figure 1. Lactate production by P. falciparum in vitro.

A (▽——▽), P. falciparum + normal serum;

Ad (○— — —○) P. falciparum + pyrimethamine serum;

B (▲——▲) non-infected blood + normal serum;

Bd (■-·—·—■) non-infected blood + pyrimethamine serum.

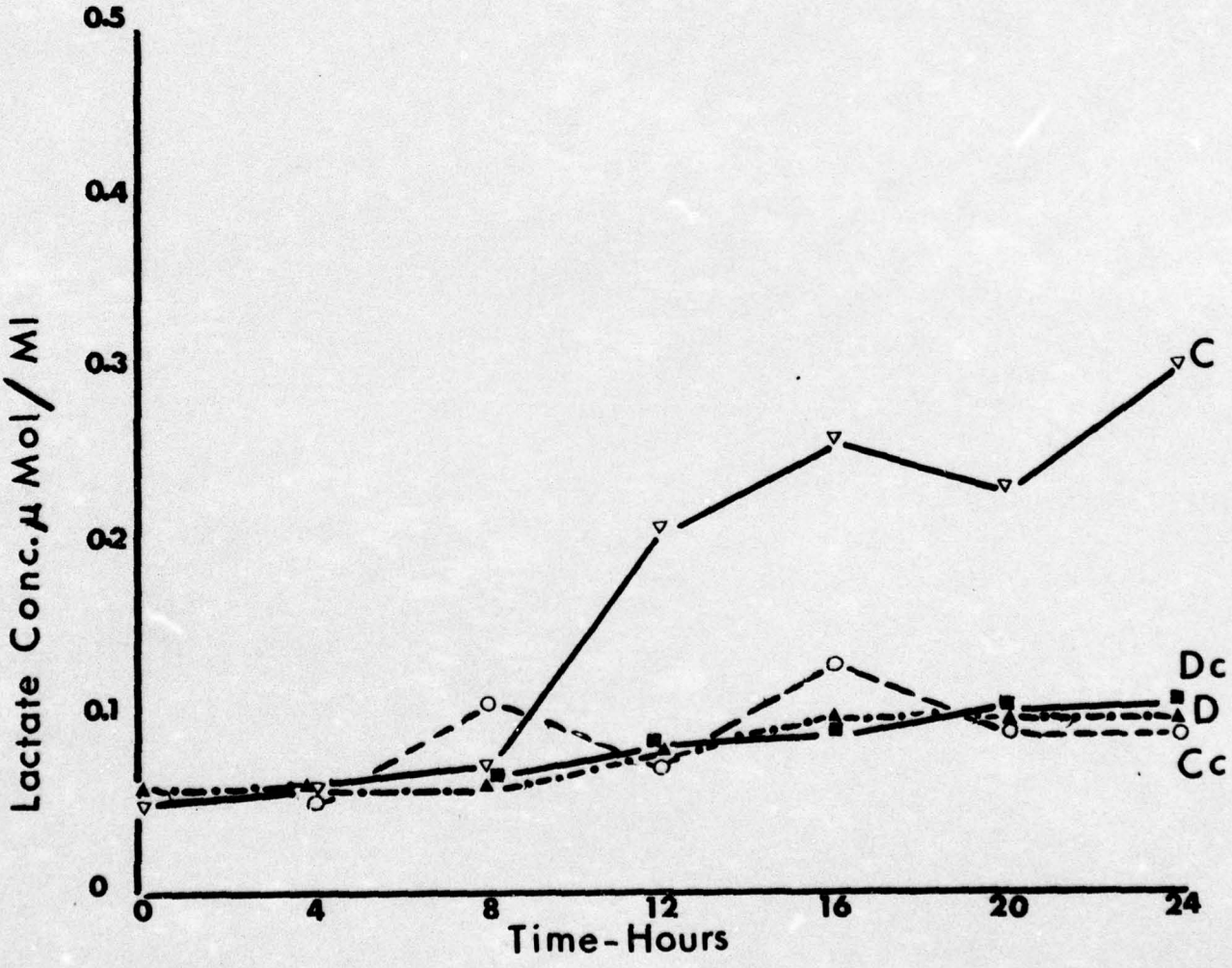


Figure 2. Lactate production by P. falciparum in vitro.

C (▽——▽) P. falciparum + phosphate buffer;

Cc (○— — —○) P. falciparum + chloroquine;

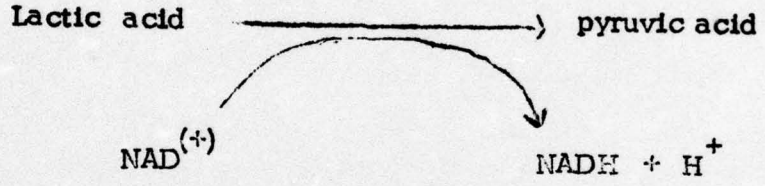
D (▲— · —▲) non-infected blood + buffer;

Dc (■——■) non-infected blood + chloroquine.

2b. Lactate production by P. falciparum in vitro

As an alternative to the use of radiotracers a number of preliminary experiments were carried out in which lactate production in vitro by P. falciparum in the presence or absence of antimalarials was monitored to see if this also provided a sensitive indicator of parasite growth and metabolic activity. The assay system for lactate is cheaper than radiotracers and simple to perform.

For these experiments P. falciparum was cultured from the large ring stage in 25 ml stopped conical flasks, each flask containing 0.2 ml resuspended blood in 2 ml supplemented TC 199. Controls were set up with non-infected adult European blood (RSP). At four-hourly intervals cultures were removed and thin blood smears made. To 2 ml of each culture 2 ml IM Perchloric acid was subsequently added. This was then neutralized by 6 M potassium carbonate. After centrifugation to remove the precipitate, the supernatant fluid was removed for lactate estimation. The standard assay procedure for lactate depends upon the reaction:



which is catalysed by lactate dehydrogenase (see Coombs & Gutteridge, 1976). 25 µl of each supernatant was found to be sufficient for the assay. Extinction was read on a Pye Unicam S.P. 600 Spectrophotometer at 340 nm.

Three experiments were carried out and the results are briefly described.

In the first experiment, one hour after the cultures were set up, 0.2 ml of serum from a donor (RSP) taking pyrimethamine was added to each of half the cultures with infected blood and half the non-infected blood controls. The remaining cultures received 0.2 ml human serum lacking pyrimethamine. Individual cultures were then processed for lactate estimations at 4 hourly intervals over the next 24 hours. Lactate production is shown in Figure 1. Irrespective of the presence of the pyrimethamine containing serum the lactate production continued in all P. falciparum infected cultures. Morphological examination did suggest that where pyrimethamine was present, schizont development was behind that of the infected controls.

In the second and third experiments serum from a donor taking pyrimethamine was replaced by adding chloroquine phosphate in phosphate buffer to a final concentration of 1.4 n moles/ml of culture 4 hours after the start of the cultures. The lactate production in one of the experiments is shown in Figure 2: the results of the two experiments were the same. Addition of chloroquine caused a rapid cessation of lactate production and examination of stained blood smears from the cultures showed that the parasites had made no growth in the 4 hours after chloroquine was added and were clearly dead 8 hours after chloroquine. Control parasites grew normally up to schizonts when the experiment was terminated.

Table 7

^{3}H-isoleucine incorporation in Ficoll-Hypaque (FH) fractionated blood

Time culture terminated	c.p.m. x 10^{-3}	
	Unfractionated blood	FH blood
Day 0 1430 hrs	0.6 ; 0.6	0.4 ; 0.4
Day 0 2245 hrs	27.9 ; 32.2	25.3 ; 28.9
Day 1 1400 hrs	192.3	186.0
Day 2 0830 hrs	113.7	139.5

These preliminary experiments confirmed that lactate production is a useful indicator of the metabolic activity of P. falciparum in vitro. Further, because the parasite produces so much lactate in vitro it was necessary to use only 25 µl of the supernatant from each 2 ml culture after perchloric acid and potassium carbonate treatment. It will be practical, therefore, to carry out further studies on lactate production on a smaller scale in the micro-cultures, and if it does prove possible to detect consistently small alterations in the level of lactate production, monitoring lactate production may provide a cheap and useful tool for the study of the effects upon parasite metabolic activity and growth of agents, such as immune sera.

3. Growth of P. falciparum in vitro after removal of white cells from the infected blood

It is planned this summer (1976) to examine again lymphocytes and macrophages for anti-P. falciparum activity in vitro. Recent reports suggest that the killing of malaria parasites by lymphocytes might occur (Coleman et al., 1975) in spite of some negative results by earlier workers (e.g. Phillips et al., 1970 ; Cohen & Butcher, 1971). Where P. falciparum infected blood and immune white cells come from different donors, in order to prevent a mixed lymphocyte reaction between the immune lymphocytes and those in the parasitized blood, it will be necessary first to remove white cells from the infected blood. The procedures used for this purpose could affect the viability of the parasite and reduce their ability to grow in vitro. Two experiments were carried out to see whether this was the case. The leucocytes were removed in two steps. In the first step the heperinized infected blood was layered onto a Ficoll-Hypaque mixture and centrifuged. The red cells and granulocytes move to the bottom of the tube leaving the monocytes at the interphase of the plasma and Ficoll-Hypaque. For the second step the granulocytes and red cells were resuspended in balanced salt solutions (BSS), run into a cotton-wool column, and incubated at 37°C for 30 min. The red cells were subsequently removed by running BSS through the column, washed once with TC 199/Hepes and resuspended to the original blood volume in autologous serum or AB serum. Examination of thick blood films from the treated blood showed an almost complete absence of white cells of any description.

After removal of the white cells the parasites were put into culture and their growth compared with that of a sample of the same infected blood from which white cells had not been removed. In the first experiment the parasites were grown in bulk culture in one-litre flasks on a shaking water bath where growth was monitored by examination of blood smears. Growth from small rings through one complete cycle was normal in control and white cell free blood. In the second experiment the white cell free (fractionated) blood and unfractionated blood were grown in microcultures and their growth followed by ^{3}H-isoleucine incorporation. The fractionated blood and non-fractionated infected blood were washed and resuspended in AB serum. Each microculture contained 10 µl infected cells, 150 µl supplemented medium and 50 µl AB serum. Cultures were set up at 1215 hrs on day 0, starting parasitaemia of 7.2 rings/100 red cells, and 1 µCi ^{3}H-isoleucine (specific activity 26 Ci/m mol) added to each well. Single or duplicate cultures were sampled for incorporation as indicated in Table 7. From stained blood smears it was seen that schizogony occurred between 1400 hrs on day 1 and 1730 hrs on day 2.

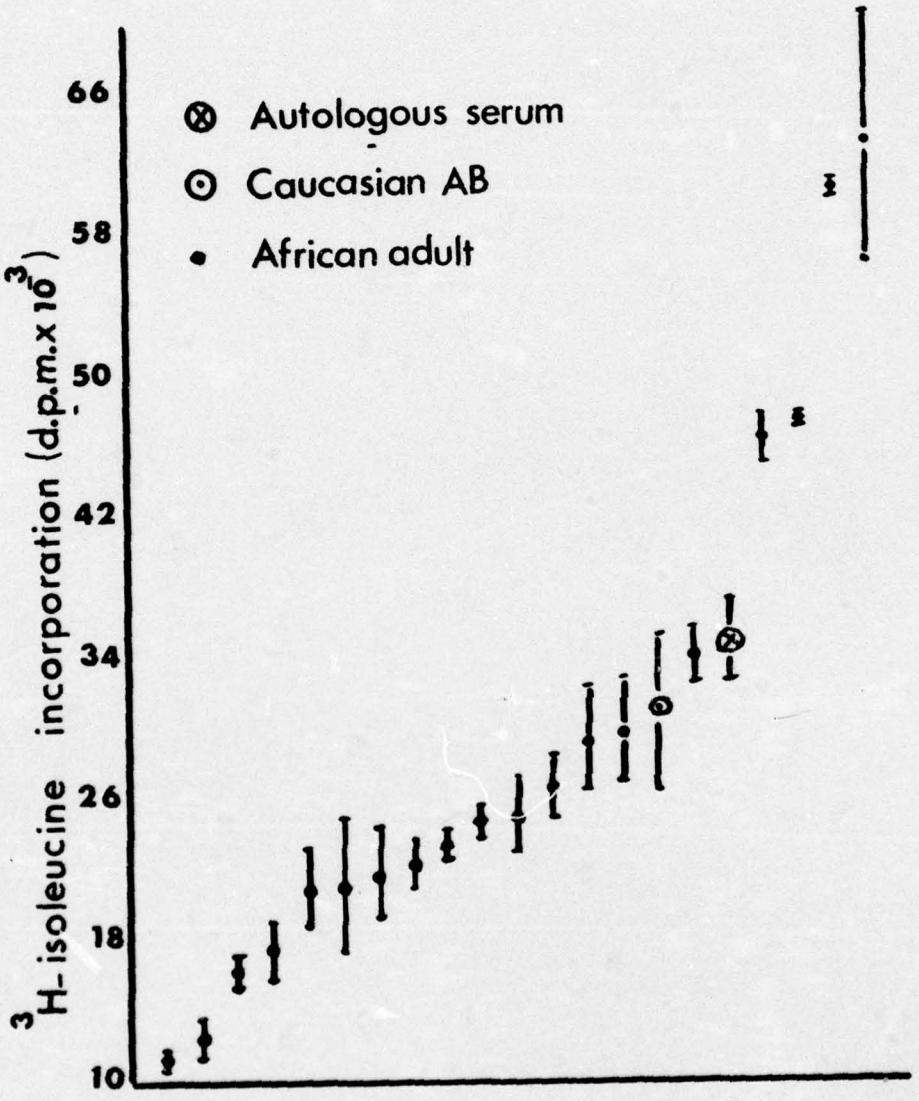


Figure 3. Incorporation of ^{3}H-isoleucine by P. falciparum parasites grown through schizogony in vitro in the presence of sera from 21 different donors. Results with individual sera, arranged according to mean isotope incorporation (range of values is also shown).

Table 8

Distribution of 11 adult sera according to the degree of inhibition they produced when tested with 4 different isolates of P. falciparum

Degree of inhibition of parasite multiplication	Different isolates of infected red cells			
	1	2	3	4
None	3	0	11	1
Up to 20%	5	1	0	4
Up to 40%	3	2	0	5
Up to 60%	0	8	0	1

Table 9

Inhibitory activity of sera tested against two broods of P. falciparum in the same blood sample

Serum	Multiplication as % of that in autologous serum	
	Brood	
	1	2
S1	103.8	43.7
S3	70.8	77.9
S4	38.5	22.1
S5	80.8	6.3
S16	88.5	52.6
S17	78.0	37.8
K331	34.6	31.6
K826	107.7	46.3
C42	100	100
RSP	110.8	102.6

C42 = autologous serum

4. Growth of P. falciparum in sera from Gambians and Nigerians

Further Gambian sera and Nigerian sera from Kano (supplied by WHO) were screened for anti-P. falciparum activity in the manner described previously (Annual Report: Contract DAJA 73-37C-3492). In brief ring stage parasites were grown through schizogony in microcultures containing in replicate 150 µl supplemented TC 199 and 100 µl of the following sera: A, Caucasian AB serum, B, serum from the donor of the infected red cells; C, serum from unrelated Gambians and Nigerians (matched for the ABO blood group of the infected cell donor). Parasite multiplication was determined by microscopical examination of stained blood smears of the cultured blood taken after reinvasion had occurred and parasite growth in some experiments was measured by H^3-isoleucine incorporation. The isotope was usually added just after schizogony and hence it was incorporated by those parasites which reinvaded and made growth before the experiment was terminated.

The inhibitory activity of a random sample of 19 adult sera is shown in Figure 3, in terms of ^3H-isoleucine incorporation. Most of the sera inhibited parasite multiplication and correspondingly there was a reduced level of isotope incorporation. Four of 19 sera enhanced multiplication and incorporation.

Well over 100 sera from Gambians of all ages have been tested, many of them against parasites from several different donors. In approximately 71% of the sera the parasites' multiplication rate was more than 10% less than that in serum from the donor of the parasites but only 31% of the sera reduced the multiplication rate by 50% or more. A sample of 86 sera from Kano, Nigeria; tested against Gambian parasites gave approximately the same results. Considering that the majority of the sera came from individuals who would be considered to be strongly immune to P. falciparum the degree of inhibition for many sera might have been expected to be higher. In addition, there was variability in the amount by which an individual serum inhibited the multiplication of parasites from different children. Table 8 summarises the data from an experiment to illustrate this point in which 11 sera from Gambian adults were tested against parasites from four different infected children.

These findings raise questions about antigenic diversity in P. falciparum as strong inhibitory activity might depend on correct matching of parasite (antigenic) type and antiserum. This variable was not controlled in the experiments described above nor in the earlier studies (Phillips et al., 1972, 1975; Mitchell et al., 1976). We therefore devised a procedure which tests known homologous or heterologous combinations of parasitized cells and sera and this is described in the next section (see below).

Two additional observations on the inhibitory activity of Gambian sera can be mentioned briefly.

A small number of adult Gambian sera were tested for inhibitory activity against each of two broods (populations) of parasites present in the same sample of infected blood (see Section 1 above). In the example given (Table 9) the two broods were 24 hrs apart in their growth and schizogony and reinvasion of each brood was distinct. The multiplication rate for each brood for each serum is expressed as a % of that in autologous (i.e. serum of

Table 10

Effect of increasing volume of serum in microcultures

Serum	% parasitaemia after reinvasion		
	Volume of serum added		
	100 µl	150 µl	200 µl
Autologous	11.3	5.0	2.3
K924	6.1	3.4	1.9
K160	16.7	10.0	6.6
S18	8.2	4.8	2.3

parasite donor). It can be seen that sera inhibitory to the first brood (large rings at start of the experiment) were also inhibitory to the second (small rings at the start) and that some sera not inhibitory to the first were so to the second. Reinvasion was equally successful in autologous and Caucasian sera for both broods. It is probable that the two broods emanate from sprozoites from at least two mosquitoes and therefore they may be of different antigenic types. For the same reasons outlined above this might account for different sensitivities of the two broods to the same serum sample. It was significant that even in inhibitory sera the second brood of parasites proceeded to grow up to the schizont stage normally and that the inhibitory activity thus resulted from interaction of the mature schizont or released merozoites and the antiserum.

100 µl of antiserum per microculture (total volume 260 µl) appeared to be about the optimal volume for most sera for demonstrating inhibitory activity. Increasing the volume to 150 µl or 200 µl increased inhibition of reinvasion in all sera including control sera as illustrated in Table 10. For inhibitory sera a reduction in the volume per culture below 100 µl reduced the relative degree of inhibition.

5. In vitro cultures of cryopreserved P. falciparum in the presence of pre- and post-treatment serum from the donor of the parasitized blood. A method to test inhibitory antibodies to wild populations of P. falciparum parasites of potentially disparate antigenicity

In Section 4 above it was noted that the variability observed in the inhibitory activity of sera from immune Gambian adults might result from a failure to match parasite (antigenic) type with antiserum. This section concerns a method we have devised to test known homologous or heterologous combinations of parasitized cells and sera.

Heparinized blood, as well as serum, was obtained from infected children. The blood was cryopreserved in an unrefined manner in a final concentration of 10% glycerol in Ringer at -22°C ± 2°C: the serum was also frozen. Each child received a single dose of chloroquine orally (5 mg base/kg) immediately after the infected blood sample had been taken, and a second serum sample was obtained 1-3 weeks later, during which time the parasitaemia had become subpatent, the chloroquine undergone catabolism and the child presumably developed some immunity to that population of parasites. In order to compare the inhibitory activities of the first (I) and second (II) serum samples, the appropriate frozen parasites were thawed, washed in medium 199, resuspended in AB serum, and grown through schizogony in microcultures in the presence of the homologous and known heterologous sera.

Studies on the metabolism of chloroquine in man (McChesney et al., 1962) indicate that a dose of 5 mg/kg should have reached the minimum therapeutic level by day 7, the earliest time we obtained any second serum samples. To check for antimalarial activity through residual chloroquine, we carried out cytotoxic tests with the sera in microcultures using P. knowlesi as a model system. The results showed that there was an overall reduction in the uptake of ^{3}H-isoleucine by P. knowlesi parasites grown in serum II samples but this

Table 11

Plasmodium falciparum multiplication in serum obtained before (I) and after (II) anti-malarial treatment. (Expressed as % of multiplication in homologous serum (I))

System	Serum	Cells	(I)	(II)	
Homologous	7529	7529	100	(12)*	19
	7536	7536	100	(14)	11
	7540	7540	100	(14)	45
	7544	7544	100	(14)	219
Heterologous	7519	7529	102	(14)	20
	7523	7529	52	(14)	28
	7525	7529	42	(7)	26
	7519	7536	56	(14)	2
	7529	7536	113	(12)	4
	7519	7539	136	(14)	56
	7523	7539	133	(14)	122
	7525	7539	96	(7)	67
	7529	7539	157	(12)	67
	7536	7539	135	(14)	102
	7506	7540	52	(14)	17
	7531	7540	146	(14)	108

Triplicate tissue cultures each contained 10 µl blood + 150 µl TC-'199' + 100 µl serum.

Percentage multiplication was calculated from the mean results.

[* Figures indicate number of days between anti-malarial treatment and collection of serum (II).]

Table 12

Uptake of ^{3}H-isoleucine (cpm x 10^{-3}) by P. falciparum grown in serum obtained before (I) and after (II, III) antimalarial treatment

System	Serum	Cells	AB	I	II		III	
Homologous	7525	7525	1.31	3.21	(7)*	0.23	(24)	2.09
	7531	7531	ND	3.32	(14)	0.05		
	7544	7544	2.25	5.98	(14)	12.84		
	7580	7580	1.46	2.54	(7)	0.28		
	7584	7584	1.76	2.19	(7)	0.73		
Heterologous	7569	7584	1.76	4.13	(13)	1.08		

Duplicate tissue cultures each contained 10 µl blood + 150 µl TC-'199' + 50 µl serum + 10 µl ^{3}H-isoleucine (0.5 mCi/ml). Incorporation values are given as means.

*Figures indicate number of days between antimalarial treatment and collection of serum (II) or (III).

ND = not done.

was not significant at the 5% level. The few ($^{5}/31$) serum II samples that had a marked inhibitory effect on P. knowlesi did not consistently inhibit P. falciparum nor were they among the samples taken only seven days after the antimalarial treatment. The residual inhibitory activity in these sera, therefore, may possibly be accounted for by factors other than residual chloroquine or its active metabolites.

Data on multiplication of P. falciparum parasites in children's sera taken just before (I) and after (II) antimalarial treatment are shown in Table 11. Parasite multiplication is given as a percentage of that in homologous (I) serum: figures greater than 100% therefore indicate enhanced growth of the parasite in homologous II or heterologous sera.

Marked inhibition of parasite multiplication occurred in three of the four examples shown in the table: in the fourth (7544) parasite multiplication was enhanced in the homologous return (II) serum, which at this stage is difficult to explain: it may possibly be because of a nutritional factor. In addition, in the table are the results of testing four parasite isolates against heterologous I and II sera. Most of the convalescent sera (II) were markedly inhibitory (t-test for 12 paired (non-independent) samples gave $P < 0.001$). In contrast in heterologous I samples inhibition occurred in only $^{4}/12$ cases (some cross inhibition might be expected because all the children were exposed repeatedly to reinfection and hence exposure to many parasite populations (antigenic types). Other noteworthy points in the table are that 7536 II serum was inhibitory to the homologous parasites but not to heterologous parasites 7539 and that 7523 II serum inhibited 7529 parasites but not 7539. In a small number of cases the growth and multiplication of the parasites in homologous or heterologous sera was measured by ^{3}H-isoleucine incorporation with similar results as shown in Table 12. It can be seen that serum 7525 (III - third sample) taken 24 days after treatment was less inhibitory than serum II obtained 7 days after treatment. Serum (II), however, was not inhibitory in the P. knowlesi cytotoxic assay. In contrast serum 7544 (II) was inhibitory in the P. knowlesi assay but caused an increased multiplication and isotope uptake (Table 12) by homologous P. falciparum parasites.

In summary our results so far show that most of the sera from children after treatment, when the parasitaemia had at least become subpatent, were inhibitory and both cross-reactive and specific inhibition was observed. The individual roles of antiparasitic antibodies, auto-antibodies especially to red cells, which may possibly be present and non-antibody effects which promote or depress parasite growth and multiplication, all need further investigation.

The extensive studies of Wilson et al. (1969) on antigens and Carter & McGregor (1973) on isozymes of wild populations of P. falciparum in the Gambia all suggest that there are mixed 'types' of this parasite. We have devised a procedure which accounts for this heterogeneity and enables us to test the homologous inhibitory antibody response to a mixed population of human malaria parasites. The methods we used to cryopreserve and to reconstitute parasitized erythrocytes were crude but further work using 10% DMSO as the cryoprotectant has improved this aspect of the test.

Summary and Conclusions

Experiments on animal malaria have shown that a number of different immune mechanisms may be involved in the control and elimination of the blood stages of Plasmodia. These include direct cytotoxic antibody activity (Cohen & Butcher, 1970), opsonizing antibody (see e.g. Brown et al., 1970), and immune spleen cells cytotoxic with and without specific antibody (Coleman et al., 1975). For human malaria the passive transfer studies of Cohen et al. (1961) demonstrated the protective role of serum antibodies and that schizonts and/or merozoites were the vulnerable stage. It is clear that for more details of immune mechanisms in human malaria we will have to rely on in vitro procedures. In vitro correlates of cell-mediated immunity are indicated a possible role for thymus dependent lymphocytes (Kass et al., 1971; Wyler & Oppenheim, 1974), in human malaria, but it is not known through which of the numerous possible ways is their anti-parasite activity mediated. More recently Wyler (1976) described an increase in null lymphocytes in convalescent children, cells which can with the help of specific antibody have cytotoxic activity to some target cells. Demonstration of, for example, cytotoxic lymphocytes in patients immune to malaria will almost certainly depend on observation of their activity on the growth and survival of the malaria parasite in culture.

The procedure described in this report in which the asexual blood stage of P. falciparum in infected human red cells can be grown on a microscale through $2\frac{1}{2}$ cycles in vitro with a mean multiplication rate after the first cycle of about three times, provides the basis for a number of investigations of the possible immune mechanisms leading to parasite death. Selection of synchronous infections makes estimation of the multiplication rate straightforward. Preliminary attempts to improve the multiplication rate in the microcultures by replacing some of the medium or adding fresh red cells did not noticeably improve the multiplication rate. Recently Trigg & Shakespeare (1975) reported that rhesus monkey red cells maintained in culture for 24-48 hrs at 37°C were less readily invaded in culture by P. knowlesi than were fresh red cells. They attribute this to changes in the integrity in the red cell membrane. If similar changes occur in human red cells in vitro then the addition of fresh red cells after 24 hrs culture might improve the multiplication rate: this aspect requires further investigation.

The use of ^{3}H-isoleucine for monitoring growth and multiplication of P. falciparum in the microcultures was an effective and sensitive rechnique. The procedure we used for measuring incorporation was laborious but had the merit of giving a high degree of consistency between replicates. Nevertheless we plan to try and simplify the procedure without sacrificing accuracy. Measuring growth of the parasite by determining levels of lactate produced is also to be examined further: this procedure has the advantage of being relatively cheap as well as sensitive.

To date the microculture technique for P. falciparum has been used for detecting the ability of Gambian and Nigerian sera to inhibit reinvasion of red cells after shizogony. The majority of sera, from individuals of all ages, and many of whom would be clinically immune, exhibited a small degree of inhibition but only a few were strongly inhibitory. Some sera inhibited parasites from some children but not others. This variability in activity could be

attributed to antigenic diversity in P. falciparum coupled with the fact that the specific antibody response to any antigenic type might only remain at a high level for a relatively short period. It is known that in rats, for example, the protective antibody levels wain rapidly in the absence of the stimulating parasitaemia (Phillips & Jones, 1972). It was therefore clear that it was necessary to match the antiserum with a particular population of P. falciparum parasites in order to circumvent the heterogeneity of the parasite. This we did by cryopreserving infected P. falciparum blood while the infected child after treatment mounted an immune response to those parasites. Subsequently we tested the preserved parasite after thawing them and putting them into culture, against pre-treatment and convalescent serum. The method of cryopreservation was crude and has since been refined using P. knowlesi as a model. Nevertheless in the tests described the cryopreserved parasites after thawing grew from rings through one cycle (see also Pavanaud et al., 1974; Diggs et al., 1975) and the convalescent sera showed both specific inhibition of homologous parasites and some cross-reactivity with parasites from other children. We consider that our method using cryopreservation to match parasites with immune response provides a practical approach to the analysis of anti-parasitic antibodies and other immune mechanisms, e.g. role of cytotoxic lymphocytes in human malaria.

The observation that cryopreserved P. falciparum to resume growth subsequently in culture, first made by Pavanaud et al., (1974), has an immediate and considerable implication, namely that with the cooperation of infected individuals, infected blood can be collected and stored deep frozen until required for use in the wide variety of investigations that can be carried out using in vitro culture of human malaria. For example, the microculture system of P. falciparum is very suitable for drug-screening. The need for expensive Aotus monkeys as laboratory hosts for human Plasmodia will be reduced. It is feasible to transport deep frozen infected blood from an academic area to distant laboratories, e.g. from The Gambia to the U.K.

PART II

IMMUNITY TO RODENT MALARIA PARASITES

Introduction

There have been relatively few experimental investigations on human malaria parasites because these parasites are very host specific and will not infect the usual range of laboratory animals. Consequently much research is carried out on model systems in which malaria parasites of wild animals are studied in laboratory animals. It is hoped that the results obtained in these systems are relevant to the human situation. In the present study immunity to the blood stage of malaria parasites isolated from thicket rats, Thamnomys spp., in West Africa are being studied in laboratory mice. The work described in this report is a continuation of the study described in the Annual Report for Contract DAJA 37-73-C3492. Four lines of investigation have been carried out:

(a) antigenic variation by P. v. chabaudi;

(b) immunosuppression by acute malaria and babesia injections;

(c) immunization of mice against murine malaria;

(d) investigation of the type of lymphoid cells involved in immunity to murine malaria.

Most of the effort this year has been devoted to the investigation of the role of different lymphoid cell types. The results from each area of investigation will be described and the conclusions arising from each will be drawn together in a concluding section.

Material and methods - general considerations

Parasites: two rodent plasmodia have been studied: Plasmodium vinckei chabaudi (A/S strain) and P. berghei (KSP11 strain). P. v. chabaudi was isolated from wild caught thicket rats (Thamnomys rutilans) from the Central African Republic and kindly supplied after cloning and mosquito passage by Dr D. Walliker, Institute for Animal Genetics, University of Edinburgh. This strain is, as far as is known, not contaminated with Eperythrozoon. In C57Bl and NIH mice of 6 weeks and older this parasite produces a primary patent parasitaemia lasting 10-14 days with a peak parasitaemia in the range of 25 to 75%. After the primary parasitaemia, apart from occasional short-lasting periods of patency, the parasite remains subpatent for about 6 weeks when the majority of mice achieve a sterile cure. In our laboratory the asexual blood stage of the parasite grows synchronously, schizogony occurring around midnight. Late trophozoite and schizont stages tend to leave the peripheral circulation.

P. b. berghei KSP11 strain was isolated from Thamnomys surdaster (Congo tree rat) from Katanga (Zaire). The parasite was kindly supplied by Dr J. Bafort from the Liverpool School of Tropical Medicine and Hygiene. It was treated with neoarsphenamine (Voller & Bidwell, 1968) soon after arrival in order to ensure that it was free of Eperythrozoon.

The parasites were stored either at -70°C in dry ice or at -198°C in liquid N_2 using glycerol at a final concentration of 10% as a protective agent. Routinely parasite populations were subpassaged every 3 days in mice when not in low temperature storage. Parasitized blood taken from the deep freeze was rapidly thawed in cold tapwater and inoculated into mice. Subpassages were subsequently made every 3 days until a patent parasitaemia had developed. In the case of P. v. chabaudi, where possible, all experiments were started from material one passage away from the mosquito population.

Heparin at a final concentration of 17 i.u./ml whole blood was used as the anticoagulant and dilutions of infected blood were made in Hanks' balanced solution.

Parasitaemias were assessed by examination of tail blood smears stained with Giemsa's stain and recorded as parasitized red cells/10^4 or 10^5 red cells. Mice were injected with parasitized red cells intraperitoneally (i.p.) or intravenously (i.v.) as indicated in the text. All infections were initiated with infected red cells.

Mice

Four strains of mice were used at different times, namely C57Bl, CFLP, CBA and NIH. The greater part of the study was carried out with CFLP, C57Bl, and NIH mice. CFLP are outbred and NIH are inbred and both strains were supplied by Carworth Europe. C57Bl are inbred and supplied by the Centre for Tropical Medicine, Easter Bush, East Lothian. An inbred CBA colony is maintained in the Zoology Department, University of Glasgow.

All animals were fed on Oxoid diet 41B and food and water were given ad libitum. They were maintained at 23°C with 12 hrs light from 0800 to 2000 hrs.

A. Antigenic variation

A characteristic of many malaria infections including the human malarias is that they persist for a relatively long time in the blood phase of the disease in spite of the host's immune response to them. It is thought unlikely that this persistent blood phase in more than a very few species of mammalian malaria parasites is the result of parasites emanating from a persistent liver stage.

A chronic P. knowlesi infection becomes established in the rhesus monkey after subcurative drug therapy in the acute phase of a blood induced infection and it has been shown that during the course of this chronic infection the parasite undergoes repeated changes of its surface antigens (antigenic variation) as detected in the late trophozoite and schizont stages (Brown & Brown, 1965; Brown, Brown & Hills, 1968; Brown, 1971; Brown & Hills, 1974). By repeated antigenic variation the parasite apparently repeatedly evades the host's humoral responses to it and thereby is able to survive in a partially immune monkey. Repeated antigenic variation in the asexual blood phase as described for P. knowlesi has not been described for any other malaria parasite.

There is some evidence, however, that antigenic variation does occur in P. cynomolgi bastianelli (Voller & Rossan, 1969), and P. falciparum (Voller, 1971) and P. berghei (Cox, 1962) but that in these species it is repeated many times as in P. knowlesi has to be ascertained. It is essential to know if antigenic variation in the asexual blood phase is a general feature of malaria parasites and to relate to it any studies on the immunology of malaria. The special significance that antigenic variation might have in the development of a vaccine against the blood phase of the disease is obvious; will the vaccine need to include all the possible variant antigens of a species or not?

A major drawback in demonstrating antigenic variation in species other than P. knowlesi is the lack of a suitable serological test. For P. knowlesi a variant specific schizont-agglutinating antibody (SICA) is produced to each variant as it emerges in the rhesus monkey.

In the present study the possibility that the rodent malarias, in the first instance P. v. chabaudi, undergo repeated antigenic variation is being investigated. There is good circumstantial evidence that P. v. chabaudi may be capable of antigenic variation. First, the parasite in the natural host, the thicket rat, persists for many months as a chronic and frequently patent infection. In C57Bl and NIH mice the parasite persists for 6 or more weeks on average as a chronic infection after the acute primary parasitaemia but when these mice have their immune system impaired by splenectomy or 600 rads x-irradiation the parasites persist as a repeatedly relapsing parasitaemia.

Previous experiments in this study showed that the 'breakthrough' population in mice which had been immunized with irradiated parasitized cells and then challenged with the same population (antigenic type) of P. chabaudi as was used for immunization appeared to be a variant of the immunizing population. This suggested some degree of antigenic lability in this strain of P. v. chabaudi. In the immunized mice there was a delay in the onset of the patent parasitaemia of the 'breakthrough' population compared with the controls. This delay is presumably, in part at least, the result of the activity of protective antibody induced by the immunization procedure (Briggs & Wellde, 1969), on the parasites with which the immunized mice were challenged and that the antibody in some way mediates the production of the new variant type. To examine this further a series of simple passive transfer experiments were carried out as follows. Pools of immune serum were prepared by reinfecting convalescent C57Bl mice. Non-immune mice were subsequently injected with the same population of P. v. chabaudi as that used for reinfecting the immune serum donors and immediately afterwards the infected mice received immune or normal serum i.p. The effect of the immune serum was to delay the onset of a patent parasitaemia in the recipients compared with the controls. Parasites from the patent parasitaemia in the immune serum recipients were then tested for their sensitivity (i.e. delay in onset of a patent parasitaemia) to the same pool of immune serum in further non-immune mice. It was thought that exposure to the anti-parasitic antibody in the immune serum would modify the antigenic make-up of the parasite population and promote the appearance of an antigenic type of reduced sensitivity. Results of a representative experiments are described.

Mice were injected with 1×10^5 parasitized red cells on day 0 and 2.0 ml immune or normal serum injected i.p. immediately afterwards. In the immune serum recipients the onset of a patent parasitaemia was delayed by four days over the normal serum recipients. The experiment was then repeated with the serum recipients being injected with 10^5 parasitized cells taken on day 10 ( ) from one of the immune serum recipients in the first transfer experiment. Again there was the same delay in the onset of patency in the immune serum recipients. The procedure was repeated a further two times on each occasion parasites for infection being collected from one of the immune serum recipients in the previous experiment. On both occasions there was no discernible loss in the sensitivity of the parasites to the antiparasitic activity of the immune serum. Thus exposure of a population of P. v. chabaudi on three successive occasions to homologous antiserum did not promote the appearance of a population of parasites (antigenic variant) of reduced sensitivity to the immune serum.

The appearance of serum resistant (i.e. reduced sensitivity to immune serum) populations of P. berghei (Briggs & Wellde, 1969; Briggs et al., 1968) and of Babesia rodhaini (Phillips, 1969; Roberts et al., 1972; Thoongsuwan & Cox, 1973; Roberts & Tracey-Patte, 1975) in similar types of experiments have been reported. It was therefore surprising to find that there was no change in the sensitivity of the P. v. chabaudi population. It may be significant that the P. v. chabaudi population used in our studies had been cloned and the parasite populations used by the other workers were not.

It was planned to prepare populations of P. v. chabaudi which had been shown to be antigenically distinct and then determine the effect of mosquito passage on the stability of their antigenic make-up. As a consequence of the results described above, it would appear better to examine further strains of P. v. chabaudi for antigenic variability by the methods used so far and to seek alternative methods of detecting antigenic variants of rodent malarias. For example, the indirect fluorescent antibody technique has been successfully used (van Meirvenne et al., 1975) for determining the antigenic type of individuals of Trypanosoma brucei group trypanosomes and the method used might be applicable to Plasmodium sp.

It has perhaps been too readily assumed that persistence of malaria infections in the semi-immune host, where there is no possibility of persistent liver stages, is the result of antigenic variation of the parasite enabling it to evade the host's immune response (see Brown, 1974). Other factors may prevent full expression of those immune responses necessary for the immune elimination of the parasite. It is now clear that the character, e.g. the relative emphasis on cell-mediated and humoral components and kinetics of the immune response to an antigen will change with time and are determined by such factors as the route of immunization and the amount of antigen. For example, Mackaness and colleagues (reviewed 1974), found that mice given 10^5 sheep red cells i.v. have a maximal delayed type hypersensitivity (DTH) response which decays exponentially from a peak on day 4, as the humoral response rises. Increasing the dose of sheep red cells eventually abolishes all detectable evidence of a DTH response in favour of a strong humoral response. It is thought that the suppression of the DTH response is the activity of the products of the humoral response, for example, antigen/antibody

complexes. It is therefore very likely that the individual components of the immune response to a multiplying malaria parasite and their interrelationships of these components will change during the course of the infection and that this will influence the ability of the parasite to survive and multiply at any particular time. The immunosuppressive effect of acute and chronic malaria (McGregor & Barr, 1962; Wedderburn, 1974; Wedderburn et al., 1975) which has been demonstrated for some antigens may extend to the immune response to the parasite itself and this too may promote the continued survival of the parasite. This area of discussion will be returned to at a later stage.

B. The immunosuppressive effect of Trypanosoma brucei, Plasmodium berghei, Babesia microti and B. hylomysci on the immunological response of mice to the parasitic nematodes Trichuris muris and Trichinella spiralis

It is well established that some protozoal infections can depress the immune response of a host to other antigens. Malaria and trypanosome infections are examples. The antigens commonly used in these studies have included sheep red cells, keyhole limpet haemocyanin and Salmonella flagellin (see Wedderburn, 1974). The most important implication of this immunodepression is its effect on the host's ability to respond immunologically to other infectious agents with which it may be concurrently infected. We have initiated a study in which the interaction of a number of different parasites are to be examined and at the outset we looked at the effect of a concurrent trypanosome, malaria or piroplasm infection on the immune response of mice to the parasitic nematodes Trichuris muris and Trichinella spiralis. In the tropics concurrent malaria, trypanosome and helminth infections of man are common. In previous work it was found that concurrent infections of either P. berghei or T. brucei could impair the immune expulsion of the intestinal nematode T. muris from mice (Phillips et al., 1974).

1) Interaction of Trichinella spiralis and P. berghei in mice (with Dr R.G. Bruce)

In the previous Annual Report the first results were described. In brief, it was shown that in adult CFLP mice infected with Trypanosoma brucei brucei 8 days before or at the same time as being infected with T. spiralis, the immune expulsion of the adult worms from the small intestine was delayed and that as a consequence of this fecund females were able to continue producing larvae beyond the period found in the controls and this was the probable reason for the increased numbers of encapsulated muscle larvae in the doubly infected mice. T. brucei infections also reduced the ability of mice immunized against T. spiralis to expel a challenge infection from the small intestine. Mice infected with P. berghei 5 days before, and at the same time as (but not 7 days after) a T. spiralis infection the adults again failed to expel the adult worms from the small intestine as rapidly as the controls. Preliminary experiments had also indicated that a concurrent P. berghei infection led to an increase in the numbers of muscle larvae established and that mice immunized against T. spiralis were less able to throw out a challenge infection. Further experiments have been carried out to confirm these last two observations.

Representative experiments are described.

(a) P. berghei and larval establishment in T. spiralis infected mice

Male CFLP mice, approximately 12 months old, were infected with 500 T. spiralis larvae orally and 5 x 10^4 P. berghei parasitized red cells i.v. on day 0. The malaria infection was controlled by chloroquine (5.0 mg/kg) on days 7 and 12. Controls also received chloroquine. The mean worm burdens for six T. spiralis and six doubly infected mice on day 14 was 95 (S.D. ± 49.7) and 202 (S.D. ± 83.9) worms respectively confirming that immune expulsion of the adult worms was being suppressed by the concurrent malaria infection. The remaining mice were killed on day 27 and the number of encysted larvae for each mouse determined. The mean larval count for 12 control mice was 50,900 (S.D. ± 32,300) larvae and for nine malaria infected mice 107,700 (S.D. ± 42,300). This experiment confirmed that the concurrent malaria infection not only delayed expulsion of the adult T. spiralis from the gut but also led to an increase in the number of encysted muscle larva.

(b) Effect of P. berghei on acquired immunity to T. spiralis

Thirteen CFLP mice were immunized against T. spiralis over a period of weeks by three larval infections. The last infection with 450 larvae was 68 days before seven of them were injected (day 0) with 5 x 10^4 P. berghei infected red cells. Thirteen days later all 13 immunized mice were challenged with 450 larvae orally. On day 17 after P. berghei all the mice received chloroquine (5 mg/kg) and 4 days after this they were killed and the number of worms in the small intestine counted. The mean worm burden for the doubly infected mice was 28.7 (S.D. ± 27.3) with only one mouse having no worms and for the immunized mice with no P. berghei infection a single mouse carried two worms and the rest were negative.

2) Effect of Babesia microti and B. hylomysci in the immune expulsion of Trichuris muris in mice (with Dr D. Wakelin)

Babesias and other piroplasms are widespread in domestic animals and as such, should they exert an immunodepressive activity, they could exacerbate the effects of concurrent parasitic helminth infections. To examine this the effect of concurrent babesia infections in NIH and CFLP mice on the immune expulsion of the parasitic nematode Trichuris muris was investigated. Babesia microti, originally from a field vole (and supplied by Dr F.E.G. Cox) and B. hylomysci from a Congo tree rat (Bafort et al., 1970) were used. Preliminary experiments were described in the last Annual Report and since then a full account of this work has been published (Phillips & Wakelin, 1976). A brief summary of the results will be given here.

Acute babesia infections in mice delayed the immune expulsion of the nematode when the patent phase of the babesia infection coincided with the pre-expulsion period of the nematode injection (see Annual Report, 1974). Suppression of worm expulsion declined when the babesia parasitaemia decline.

Table 13

Persistence of the immunodepressive effect exerted by *Babesia hylomysci* infection on the immune expulsion of *Trichuris muris* from NIH mice

Injection	Number of worms recovered (Mean ± S.D.)				
	Day 10	Day 13	Day 15	Day 20	Day 28
T. muris only	70.2 ± 18.4	3.7 ± 7.2	0.9 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
T. muris + *B. hylomysci*	not done	31.7 ± 27.3	10.5 ± 8.0	0.2 ± 0.4	0.0 ± 0.0

Table 14

Effect of concurrent infection with *B. microti* on the expulsion of a challenge infection with *T. muris* from immune NIH mice

Group of mice	Infections	Cortisone	No. of mice with worms present on day 7	No. of worms recovered on day 7	
				Mean	S.D.
Non-immune	*T. muris* only	-	6/6	53.2	8.5
Immune	*T. muris* only	-	0/5	0.0	0.0
"	*T. muris* only	+	2/5	0.8	1.3
"	*T. muris* + *B. microti*	-	4/5	2.0	1.4
"	*T. muris* + *B. microti*	+	4/5	1.0	0.70

For example, the worm burdens at different times after infection in NIH mice infected with T. muris on day 0, some of the mice also being infected with B. hylomysci on day 2, are given in Table 13. The B. hylomysci infections were patent from day 3 to 14, reaching a peak on day 8. It can be seen that although the expulsion of the worm was delayed in the mice infected with B. hylomysci compared with the T. muris only controls, the delay was only temporary and within three or four days the persisting worms had been expelled. In contrast to the effect of P. berghei and T. brucei on acquired immunity to T. muris and T. spiralis in mice, in three experiments, it was not possible to show that acute babesia infections impaired the ability of immune NIH mice to throw out a challenge infection. In a representative experiment ten NIH mice, immune to T. muris, were infected with B. microti and ten immune mice were set aside as controls. Eight days after B. microti infection, when the parasitaemia was approximately 20%, the Babesia-infected immune mice, control immune mice and some non-immune controls were challenged with T. muris. On days 5 and 6 of the T. muris infection, five of the doubly infected mice and five immune controls were given 1.25 mg of cortisone in order to prevent loss of worms present at that time (Wakelin, 1970). All the mice were killed seven days after the T. muris infection and their worm burdens are shown in Table 14. The results show that the Babesia infection had no significant depressive effect on the acquired immunity to T. muris, although there was a small and probably very short-lived depression had been induced, as small numbers of worms persisted in the Babesia-infected mice, whereas the challenge control mice were free of worms.

Conclusions

Detailed knowledge of the basis of the immunodepression produced by the protozoa would help in the understanding of the mechanisms involved in the immune expulsion of the two nematodes. Only the implications, however, of this immunosuppression will be briefly considered here. Information on the mechanism of immunosuppression has been reviewed (Wedderburn, 1974; Greenwood, 1974; Greenwood et al., 1975). Our results show that malaria parasites, trypanosomes and babesias interfere with the acquisition and expression of immunity to nematode parasites to varying degrees, and it is likely that this interference will extend to other parasite organisms as well; for example, Cox recently (1975) showed that malaria infection can suppress the development of acquired immunity to T. musculi in mice. In man, P. falciparum (McGregor & Barr, 1962; Greenwood et al., 1972) and T. gambiense (Greenwood et al., 1974) can be immunosuppressive and therefore it can be expected that infection of individuals with these protozoa can exacerbate the effect of concurrent pathogenic helminth infections to which a protective immune response develops, e.g. Schistosoma haematobium (Bradley & McCullough, 1973).

Both trypanosomes and babesias are widespread in domestic stock, the former mainly in the tropics and the latter world-wide. In domestic stock, therefore, the potentiation of the pathogenic effects of parasitic organisms including helminths, by acute concurrent babesia and trypanosome infections is very likely and should be investigated.

Table 15

Immunization with BCG and irradiated P. berghei parasitized cells

Group	Irradiated blood injected		
	Injected[a]	Non-injected[b]	BCG[c]
1	+	-	+
2	+	-	-
3	-	+	+
4	-	+	-

a - 1.29×10^8 parasitized red cells (7.1×10^8 red cells in total) per mouse on day 0 and 2.6×10^8 parasitized red cells (8.4×10^8 red cells) on day 31.

b - 7.1×10^8 red cells per mouse on day 0 and 8.4×10^8 red cells on day 31.

c - 0.15 ml and 0.1 ml i.v. on days 0 and 1 respectively (total of approximately 5×10^5 - $\times 10^6$ viable organisms per mouse).

With regard to further investigations, two studies are proposed. First, it is proposed to examine the effect of concurrent trypanosome and malaria infections on immunity of mice to Schistosoma mansoni (with Dr J. Kusel), and secondly, to examine the reported immunosuppressive activity of T. spiralis (see Faubert, 1976) on the primary immune response of mice to P. v. chabaudi.

C. Immunization against murine malaria

i) Use of adjuvants

Two approaches are being made by different workers to the problems of immunization against malaria. One approach is to immunize susceptible animals or persons with the sporozoite stage of the malaria parasite inducing an immunity which is specific for this stage (Nussenzweig et al., 1972; Clyde et al., 1973, 1975; Rieckmann et al., 1974) and the second is to immunize against the asexual blood stage (e.g. Freund et al., 1948; Corradetti et al., 1966; Biozzi et al., 1970; Mitchell et al., 1975). Both approaches encounter a major problem in the limited availability of parasite antigen. We are using adjuvants to try and potentiate the immunity conferred on mice after injection with irradiated parasitized red cells, thereby reducing the quantity of antigen required to induce an effective immunity. BCG and Bordetella pertussis, both in the form used for human vaccination, were chosen as adjuvants.

In the last Annual Report it was reported that B. pertussis injected i.p. immediately after the first of two immunizing inocula of irradiated parasitized cells did markedly potentiate the resistance of the immunized mice to challenge. This heightened resistance was seen as a shortening of the patent primary parasitaemia and a lower peak parasitaemia. Preliminary experiments with BCG also showed that following an immunization schedule similar to that used with B. pertussis but with the adjuvant administered i.v., the adjuvant increased the resistance of the immunized mice to challenge. Further experiments with BCG have been carried out and a representative experiment is described.

Preparation of irradiated blood

P. b. berghei or P. chabaudi (see below) infected mice were bled by cardiac puncture and heparin was added to 15 i.u./ml to prevent clotting. Unless stated otherwise the heparinized whole blood was then irradiated with 40 K rads of γ-irradiation delivered from a ^{60}Co source at 200 rads/min. Before and after irradiation the blood was kept in ice. The irradiated blood was inoculated, usually i.p., into the recipient mice within two hours of irradiation. Non-infected blood was treated in the same way.

Immunization with irradiated P. berghei and BCG

Female 3 month old CFLP mice were used. Four groups of mice were set up and immunized as shown in Table 15. On day 37, six days after the second and last immunizing inoculum, seven mice from each of the four groups were infected with 5×10^4 parasitized red cells intravenously. A further six mice from each group were similarly challenged on day 49, eighteen days after the last

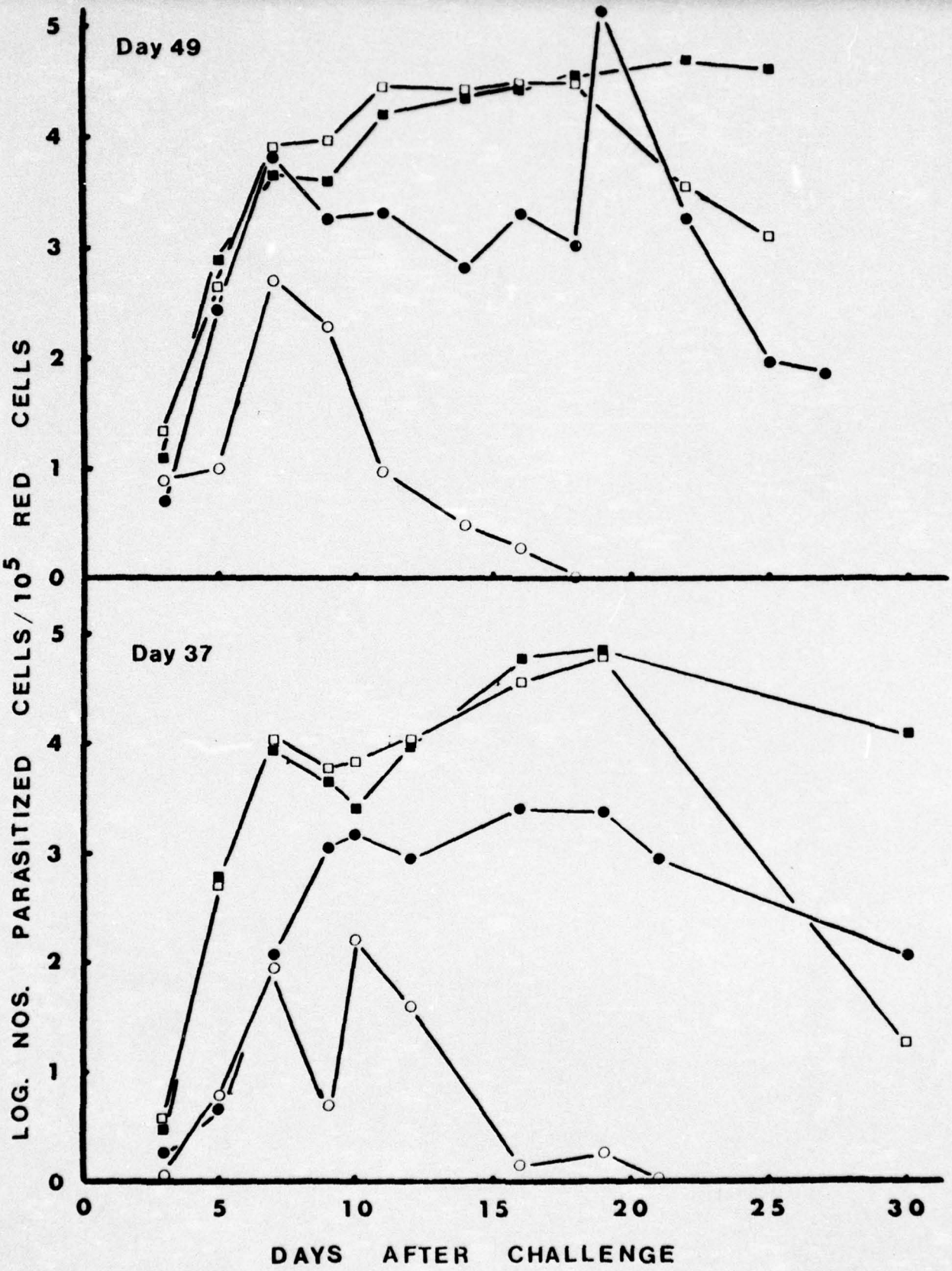


Figure 4

Immunization with irradiated P. berghei parasitized red cells and BCG. (○ ——— ○) irradiated P. berghei + BCG; (● ——— ●) irradiated P. berghei alone; (□ ——— □) irradiated red cells + BCG; (■ ——— ■) irradiated red cells alone.

Figure 5

Immunity after reimmunization with irradiated P. berghei.
(●——●) irradiated red cells; (o——o) mice
given third immunizing inoculum; (▾——▾) mice
given two immunizing inocula only.

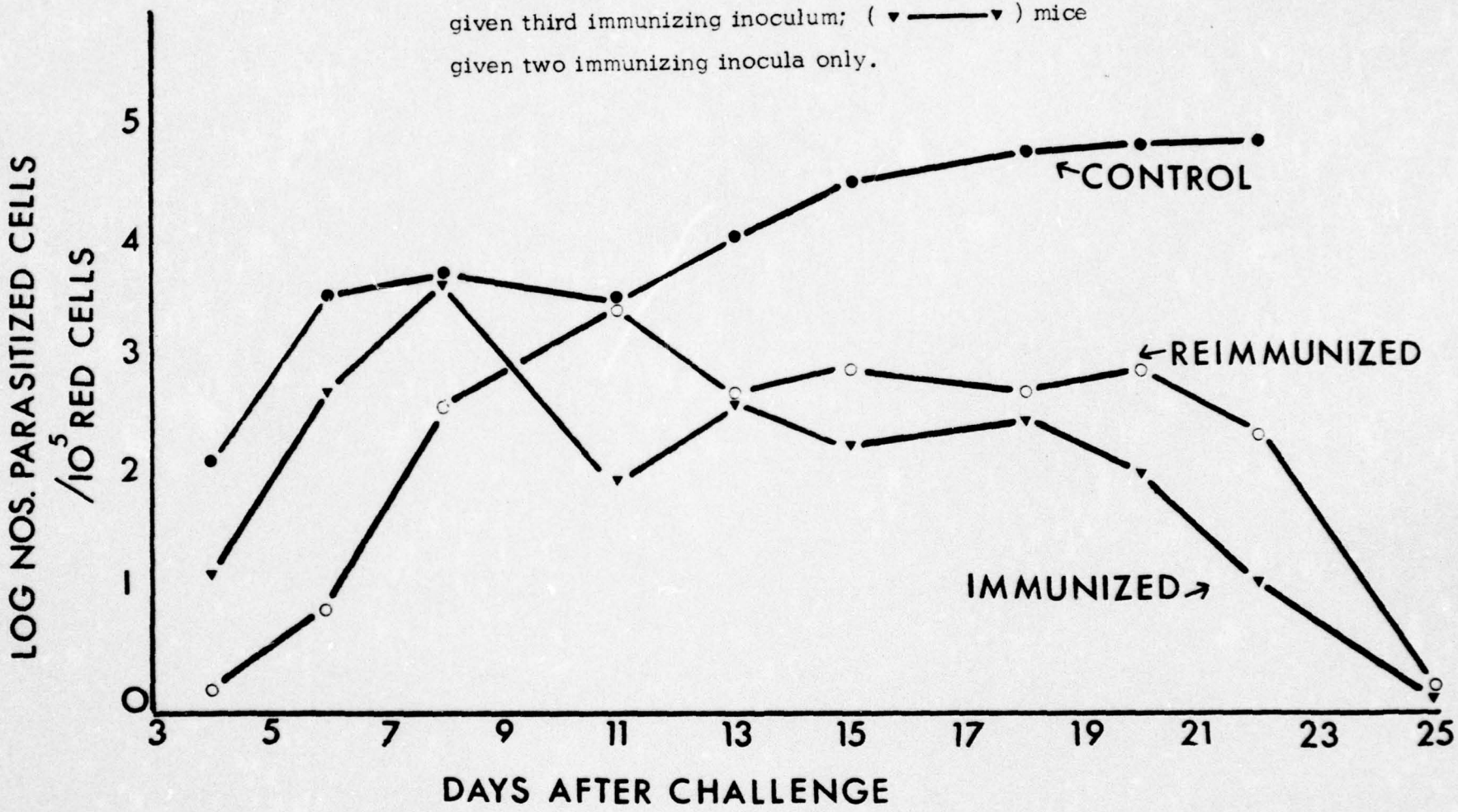


Figure 6

Immunization with irradiated P. chabaudi. (o——o) rings; (▼——▼) early trophozoites; (△——△) late trophozoites/schizonts.

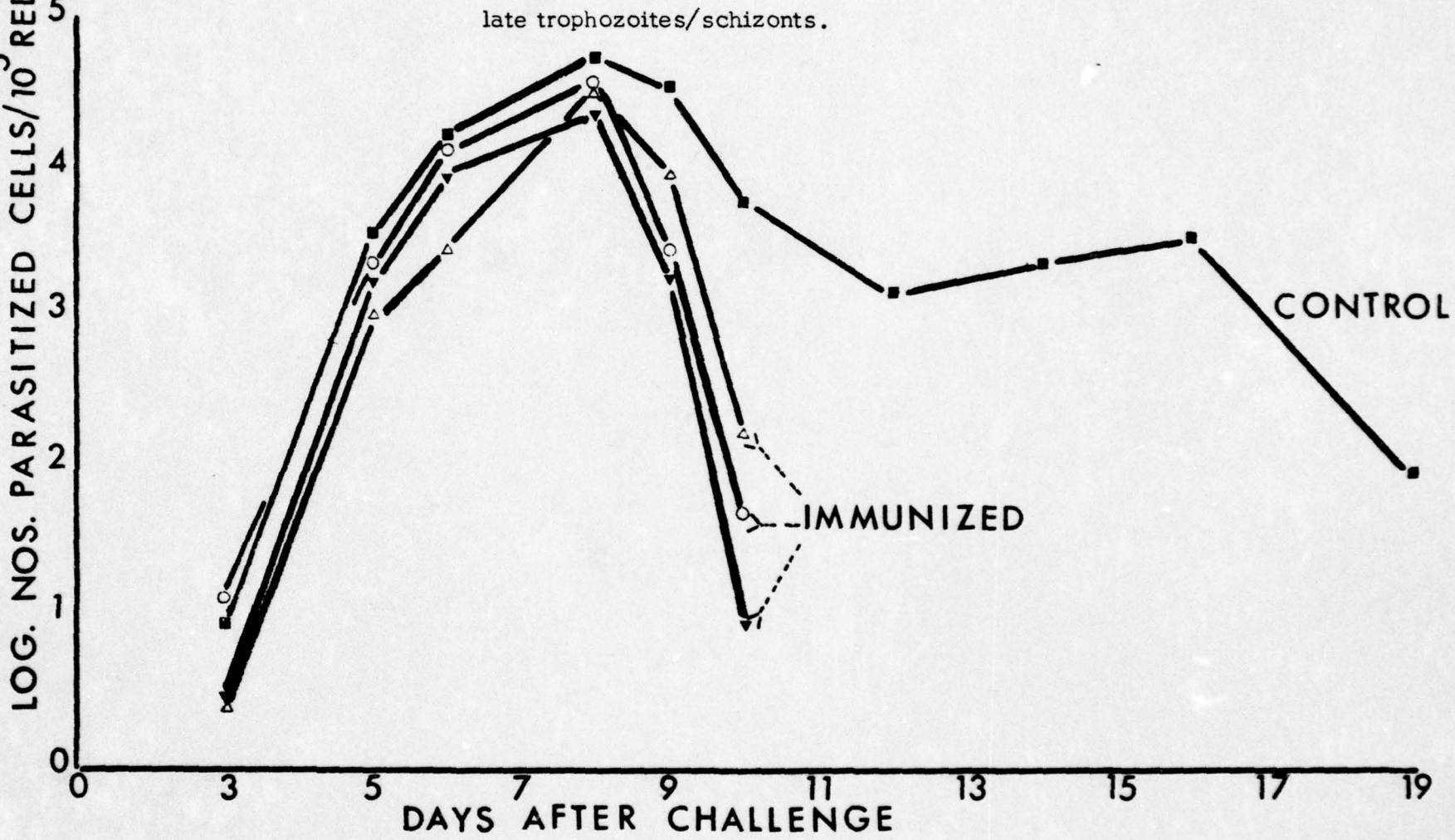


Table 16

Immunization with either rings, trophozoites and schizonts

Group	Stage immunized with	Time donors bled
1	Rings	0845 hrs
2	Early trophozoites	1500 hrs
3	Late trophozoites and schizonts	22-2400 hrs
4	Non-infected red cells	0900 hrs

In groups 1-3 each mouse received 3×10^8 P. chabaudi parasitized red cells i.p. on each occasion.

Group 4 mice received the same total number of red cells as groups 1-3 mice.

immunizing inoculum. The geometrical mean parasitaemias and mortality for each batch of six or seven mice challenged on the two occasions are shown in Figure 4. After challenge on day 37, there was a marked delay in the onset of a patent parasitaemia compared with the controls in both groups which received irradiated parasitized cells. Compared with the other three groups the patent primary parasitaemia was subsequently shortened and had a lower peak in the mice receiving irradiated parasitized cells and BCG. None of the latter group died, one of seven mice in group 2 (irradiated parasitized only), four of seven in group 3, and six of seven in group 4. After challenge on day 49, the result was essentially the same, other than that there was not a marked delay in the onset of the patent parasitaemia in the immunized groups. BCG i.v. in combination with irradiated parasitized (group 1) red cells again promoted a rapid resolution of the patent primary parasitaemia and in these mice the peak parasitaemia was well below that observed in the other groups and none of them died. All six group 4 mice died, and half the mice in groups 2 and 3.

On day 60, 17 mice remained in group 2 (irradiated parasitized cells only) and eight in group 4. Ten of the group 2 mice were injected with 5.06×10^8 irradiated parasitized cells and the remaining seven mice in this group and the eight mice in group 4 received an equivalent number of irradiated non-infected red cells. Six days later all the mice were challenged with 5×10^4 parasitized cells i.v. and the geometrical means, parasitaemias and the deaths, are shown in Figure 5. The important feature in this figure is that the onset of the patent parasitaemia in the reimmunized mice in group 2 occurs after that of the other two sets of mice as indicated by the levels of the parasitaemias on days 4 and 6. On day 4 the highest parasitaemias were in the normal red cell control mice.

ii) Immunization of mice with different stages in asexual growth cycle of P. v. chabaudi

P. v. chabaudi grows quite synchronously in the red cells of the mouse in our laboratory. Although there is a tendency for the later schizont stages to withdraw into the capillary beds, in heavily infected mice it is possible to collect infected blood containing high numbers of late trophozoites or early schizonts. The comparative immunogenicity of ring, trophozoite and schizont stages was assessed. Groups of C57Bl mice were set up which were immunized with two doses of 40 K rads: irradiated parasitized red cells seven days apart containing either ring, trophozoite or early schizont stages as shown in Table 16. Fifteen days after the first immunizing inoculum the mice were challenged with 5×10^4 P. chabaudi parasitized red cells i.v. and the geometrical mean parasitaemia for each group is shown in Figure 6. It can be seen that all the groups receiving irradiated parasitized cells were protected compared with the non-infected red cell control and the degree of protection was the same irrespective of the stage used for immunization.

iii) Comparison of the immunity derived from irradiated or heated P. v. chabaudi infected red cells

D'Antonio (1972), in a paper lacking some important technical details, reported that P. berghei infected red cells, after exposure to temperatures above 37°C for various periods of time, could act as effective inducers of immunity. If the immunity induced by heat-inactivated P. chabaudi compared

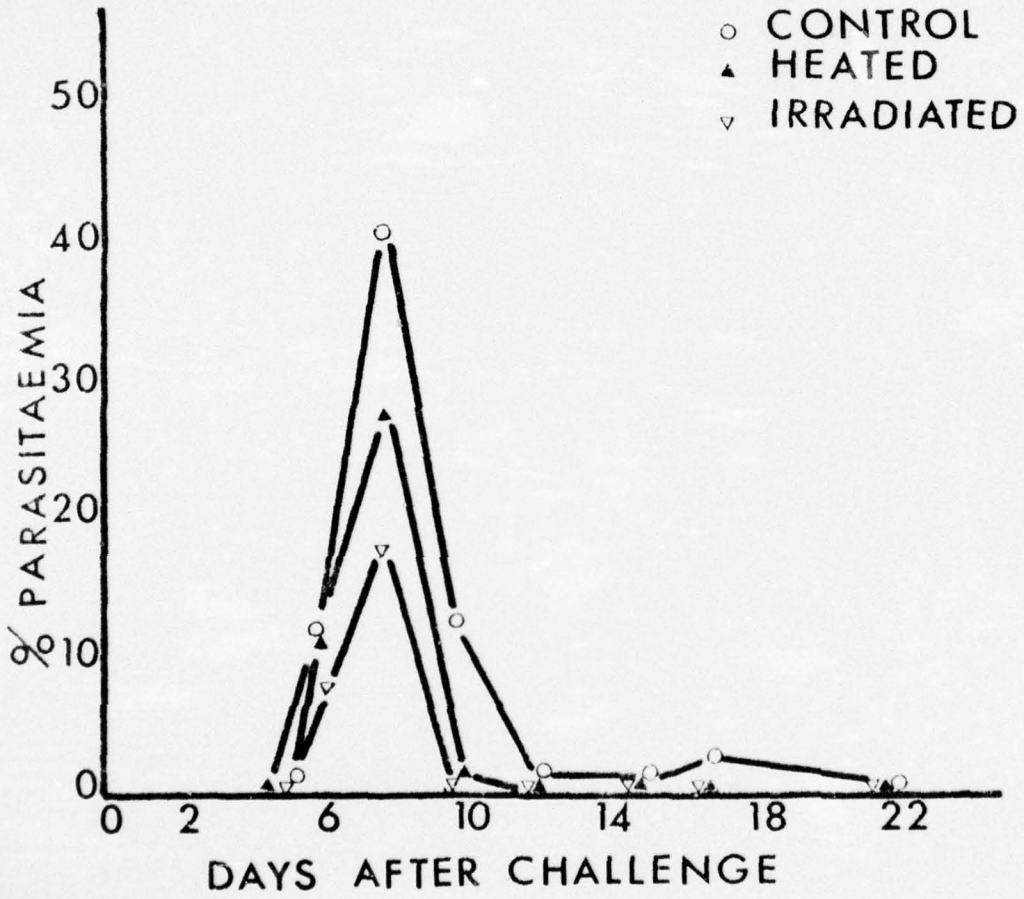


Figure 7

Immunization with heated or irradiated P. chabaudi. (o——o) heated and irradiated normal red cells; (▽——▽) irradiated P. chabaudi; (▲——▲) heated P. chabaudi.

Table 17

Immunization with heated or irradiated

P. chabaudi

Group	Immunization procedure	
	Parasitized cells*	Non-infected cells**
1	Heated	-
2	Irradiated	-
3	-	Heated
4	-	Irradiated

*3.6×10^8 parasitized cells (1.2×10^9 red cells in total) per mouse on day 0 and 1.2×10^8 parasitized cells (1.2×10^9 red cells) on day 6.

**1.2×10^9 red cells per mouse on each occasion.

well with that produced by irradiated P. chabaudi then the latter procedure was the one of choice because of its practical simplicity. We have started to examine this. The result of a preliminary experiment is given. Three month old male C57Bl mice were injected on two occasions, six days apart, with irradiated or heat-inactivated parasites (late rings) as shown in Table 17. The irradiated parasitized and non-infected cells received 40 K rads γ-irradiation and the heat-inactivated parasitized and non-infected cells were heated at 45°C for 45 min in a water-bath. All the mice were challenged with 5×10^4 parasitized cells on day 15 and the mean parasitaemias in each group are shown in Figure 7. The significant features are that in both immunized groups the primary patent parasitaemia was shortened and the peak lower than in the controls but in the mice immunized with irradiated parasitized cells the peak parasitaemia was significantly below that of the heated-parasitized cell group. The parasitaemias in both control groups followed a similar course.

Discussion and conclusions

It was confirmed that BCG exhibited pronounced adjuvant activity when injected intravenously on the same day as irradiated P. chabaudi parasitized cells. Since the last Annual Report a number of reports have appeared in the literature of the use of different adjuvants in conjunction with the blood stages of a number of Plasmodia. None of them describe the combination of adjuvant and irradiated parasitized cells. The results of different workers have varied. Desowitz (1975) vaccinated rats with a soluble extract of P. berghei incorporated in a number of different adjuvants. The rats were vaccinated with one dose of antigen and adjuvant subcutaneously and the animals were challenged 10 days later. Soluble antigen alone did not stimulate a significant degree of protective immunity but it did in combination with saponin, hexylamine, B. pertussis, levamisole, and polyinosinic-polycytidylic acid (poly I:c). The non-effective adjuvants included complete Freund's adjuvant and polyadenylic-polyuredelic acid (poly (A:U). Similarly Reisen & Hillis (1975) were unable to protect mice with foot-pad injections of freeze-thawed P. berghei infected red cells made up in emulsions of complete Freund's adjuvant (CFA). There are, however, a number of reports of the successful use of CFA in combination with blood stages of plasmodia, particularly P. knowlesi in rhesus monkeys (Freund et al., 1948; Targett & Fulton, 1965; Brown et al., 1970). Of particular note are the results of Mitchell et al. (1975) who successfully vaccinated rhesus monkeys with intramuscular injections of free merozoites of P. knowlesi in complete and incomplete Freund's adjuvant. CFA is not suitable for use in man, and hence the choice of B. pertussis and BCG in our investigations.

Hitherto there were few reports of the use of BCG in spite of its similarity in some respects to CFA. Schenkel et al.(1975) vaccinated rhesus monkeys with lyophilized P. knowlesi antigen mixed with BCG and adjuvant 65 (Hilleman et al., 1972) and injected intramuscularly. The adjuvants alone or antigen injected with BCG but without adjuvant 65 apparently gave no protection. Adjuvant 65 substitutes peanut oil for the mineral oil in Freund's adjuvant and is safely used in man. Similarly, Brown & Tanaka (1975),

using a P. knowlesi schizont infected cell antigen emulsified in incomplete Freund's adjuvant (ICFA), found that when they combined this with either Mycobacterium butyricum or BCG a proportion of the immunized monkeys were protected. BCG with antigen and no ICFA was not tried.

In spite of the results of Schenkel et al., our results suggested that further investigations with BCG were warranted and this was confirmed by the recent report of Clark et al. (1976) describing the long-lasting and non-specific anti-malarial and anti-babesial activity of large doses of BCG given intravenously to mice. These workers injected 2×10^7 viable BCG organisms i.v. compared with 5×10^5 -10^6 viable organisms used in our experiments. We found that no significant non-specific protection derived from the BCG given with non-parasitized cells but this is probably to be explained by the fact that we used a considerably smaller dose of BCG. Clark and colleagues suggest that the non-specific activity of the BCG is mediated through some factor(s), such a migration inhibition factor or interferon and not through the reported ability of BCG to increase antibody production (Miller et al., 1973) or make macrophages hyperactive (Biozzi et al., 1960). In our own experiments, with BCG, as noted above, we found no non-specific antiparasitic activity and therefore consider its potentiating activity is the product of its activity as an adjuvant for which there is, to date, no satisfactory explanation. Mackaness et al. (1974) found that BCG, given intravenously, abolished the inhibitory activity of the products of the humoral immune response of mice to sheep red cells and enabled the delayed-type hypersensitivity response of mice to this antigen to be expressed at the same time as antibody was being produced. If the same relationship between humoral and cell-mediated immune mechanisms prevails in animals infected with malaria as it does for sheep red cells in mice and that both immune mechanisms play a part in the control and elimination of the malaria parasite, then clearly immunization with BCG i.v., by promoting the expression of the two arms of the immune response at the same time, should increase the immunity of the immunized mice. It may be that in the absence of BCG the suppressive effect of the humoral response on cell-mediated responses may be a contributory factor in the persistence of the malaria parasite in the semi-immune host.

In the BCG immunization experiments it was found that the delay in the onset of the patent parasitaemia after challenge in the immunized mice compared with the controls, rapidly declined as the interval between the second (and last) immunizing inoculum and challenge increased. Boosting the mice with a third immunizing inoculum extended the prepatent period again. The delay in onset of the patent parasitaemia is almost certainly determined by the level of circulating protective antibody at the time of challenge (Briggs & Wellde, 1969), and the changes in the delay in the onset of the patent parasitaemia seen in this experiment emphasises how quickly protective antibody levels decline in the absence of the parasite (Phillips & Jones, 1972).

Irradiated rings, trophozoites and schizonts were equally effective in protecting mice. After 40 K rads irradiation the ring stages do continue to grow up to trophozoite and the irradiated trophozoites in their turn show some differentiation into the schizont stage (see Trigg et al., 1972).

Recently Mitchell et al. (1975), working with P. knowlesi in rhesus monkeys, have found that free merozoites, incorporated with Freund's adjuvant, were better immunogens than schizont infected cells. It is not known whether this also applies to other malaria parasites but their results suggest that different stages in the asexual growth cycle have different immunogenicity: our preliminary results do not bear this out although in our experiments adjuvants were not used.

The preliminary experiments assessing and comparing the resistance of mice to challenge after immunization with either irradiated or heat-inactivated P. chabaudi showed that parasites treated by both procedures were immunogenic and the mice were protected to some degree but the strongest immunity was conferred on the mice which received the irradiated parasitized cells. D'Antonio (1972) immunizing mice against P. berghei did not compare heat-inactivated with irradiated parasitized red cells but apparently the immunity from heat-inactivated P. berghei compared well with that induced by disintegrated P. berghei (D'Antonio et al., 1970). In his paper, D'Antonio (1972) does not give very full technical details which make it difficult to repeat his procedures and hence the selection of 45 min at 45°C for heat-inactivating P. chabaudi in our experiment was arbitrarily chosen. Spitalny & Nussenweig (1972) found that heat-inactivated P. berghei sporozoites did protect mice against sporozoite challenge but less well than X-irradiated sporozoites. The convenience of heat-inactivation commends it as a procedure worth further investigation.

D. Investigation of the type of lymphoid cells involved in immunity to murine (P. chabaudi) malaria

Introduction

Immunity to P. chabaudi in mice can be passively transferred with serum and spleen cells. It was noted in last year's report that phagocytosis of trophozoites, schizonts and possibly free-merozoites had been observed in the spleen and liver and occasionally in peripheral blood of immune mice after reinfection and in mice during the declining primary parasitaemia. Into the immune mice young ring stage parasites were injected i.v. and these were found to continue to grow normally up to the late trophozoite stage. During the period of growth from this stage to the completion of the asexual growth cycle the parasitaemia rapidly declined and phagocytosis of the parasites was observed. In non-immune mice the parasite grows synchronously until peak parasitaemia is reached. Then synchrony tends to be temporarily lost, a situation which to the writer appears to be the result, in part at least, of a slowing in the rate of growth of parasites after reinvasion. Many progress only to the trophozoite or early schizont stage before being lost from the circulation. Many of these trophozoites and schizonts have an abnormal morphology and presumably are similar to the crisis forms described by earlier workers (Taliaferro & Taliaferro, 1944; Brown et al., 1970; Clark et al., 1975).

After peak parasitaemia, as the parasitaemia declines, synchrony is regained and parasites are lost at the late trophozoite/schizont stage. Thus phagocytosis plays a part in the removal of the asexual blood stage and that the later stages in the asexual growth stages are the vulnerable stages. Opsonizing antibody is probably involved. In mice going through the crisis of the patent parasitaemia many of the parasites may have been dead before being phagocytosed.

In order to know more about the immune mechanisms leading to parasite death it is necessary to know which of the different lymphoid cell types are involved. We have investigated this by attempting to separate immune spleen cells into thymus-derived (T-) cells and bursa-derived (B-) cells and investigating the immunity conferred on non-immune mice after adoptive transfer of these cells to them. The method of separation was a modification of that used by Julius et al. (1973) as follows. The entire operation is carried out aseptically. Spleens were removed from the mice and collected in TC 199 (buffered with 25 mM Hepes). They were then chopped into small pieces and pushed through a stainless steel sieve into TC 199 containing 5% inactivated foetal calf serum (5% FCS/199) and heparin at 15-20 i.u./ml. The dissociated cells were subsequently deposited by centrifugation (250 g), and resuspended in 1 ml 5% FCS/199. The red cells in the dissociated spleen cell population were lysed by adding to the 1 ml of spleen cells 9 ml of a solution of Tris-NH_4Cl (one part Tris-pH 7.6 and nine parts 0.83% NH_4Cl) and leaving the cells for 5 min at room temperature. At the end of this period the cells were centrifuged, washed three times with 5% FCS/199 and finally resuspended in 2-3 ml of the same medium. The spleen cells, now free of red cells, were next run through a glass-wool column. This consisted of 5 ml of plastic syringe barrel packed tightly to the 4 ml mark with washed glass-wool. The column was flushed with 30 ml Hanks' balanced salt solution before the cells were added to the column and immediately washed through with 5% FCS in TC 199. This procedure removed clumps of cells, most dead cells and some cells macrophages. After collection from the glass-wool column the cells were centrifuged (250 g) and resuspended in 20% FCS in TC 199 to a concentration of approximately 10^8/ml. The cells were then run through a second column containing nylon wool (Travenol Labs.). This column was a 10 ml plastic syringe barrel packed to the 6 ml mark with nylon wool. The column was bathed in 20% FCS/199 and incubated for 90 min at 37°C before 2 ml of the cell suspension (10^8 cells/ml) was run into it. The cells were subsequently incubated in the column for 45 min at 37°C, and the enriched T-cell population was then eluted dropwise from the column through a 21 gauge needle as warm (37°C) 20% FCS/199 was run through. The cells in the first 25 ml of effluent were collected and then a further 20 ml of warm medium was run rapidly through by the end of which no further cells were being released from the column. The cells adhering to the nylon wool (enriched B-cell population) were released by pushing 2 x 5 ml warm 20% FCS/199 through the column with the syringe plunger. Cell viability was checked by trypan-blue exclusion (always greater than 90%) and the proportion of cells bearing Ig receptors determined using a fluorescein labelled anti-mouse Ig antibody (Flow-Labs). The cells were resuspended in 20% FCS/199 before i.v. injection into the recipients.

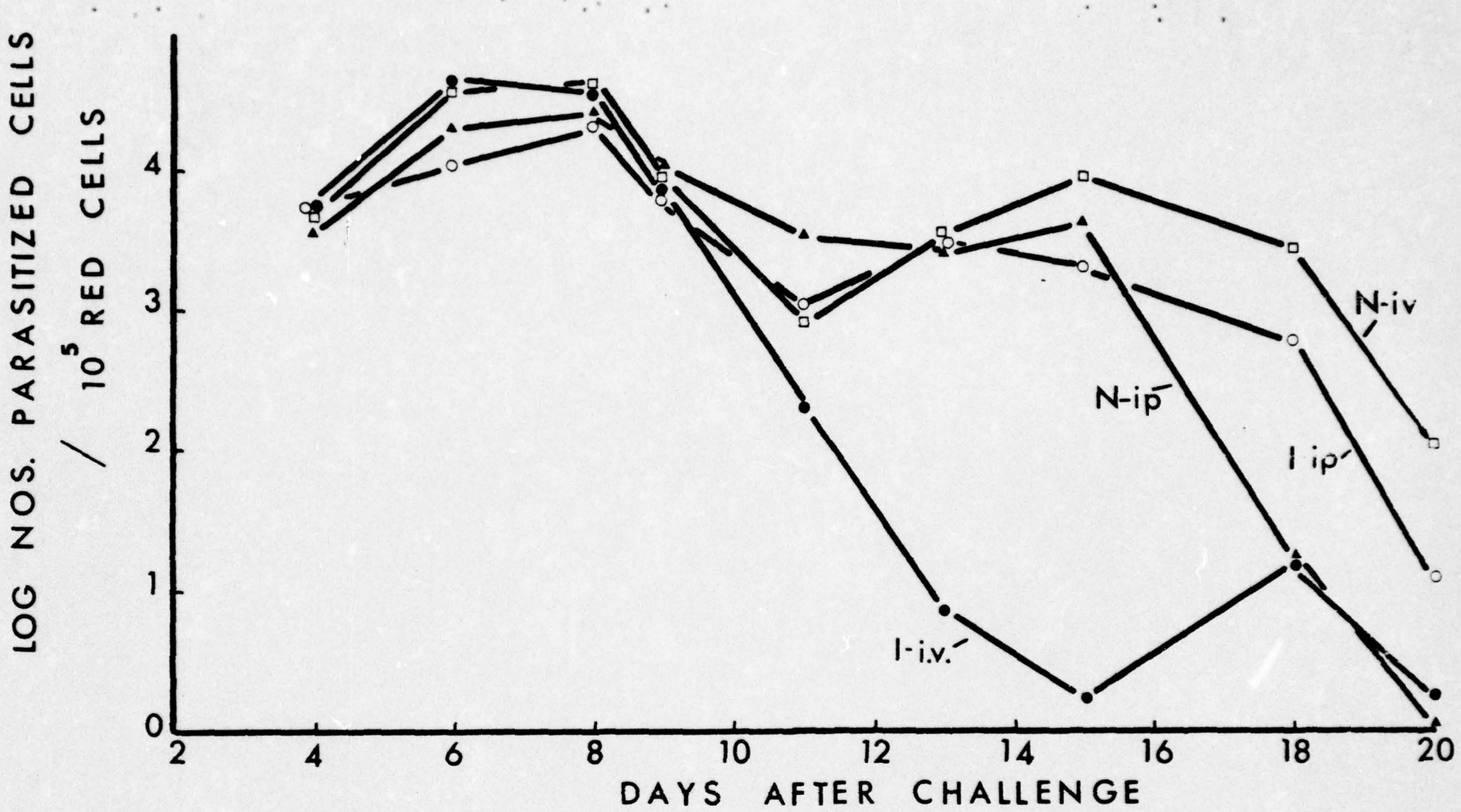


Fig. 8. Comparison of protection after immune spleen cells injected either i.v. or i.p. Parasitaemias given as the geometrical mean for each group. Immune spleen i.v. (●——●) and i.p. (o——o). Normal spleen cells i.v. (□——□) and i.p. (▲——▲).

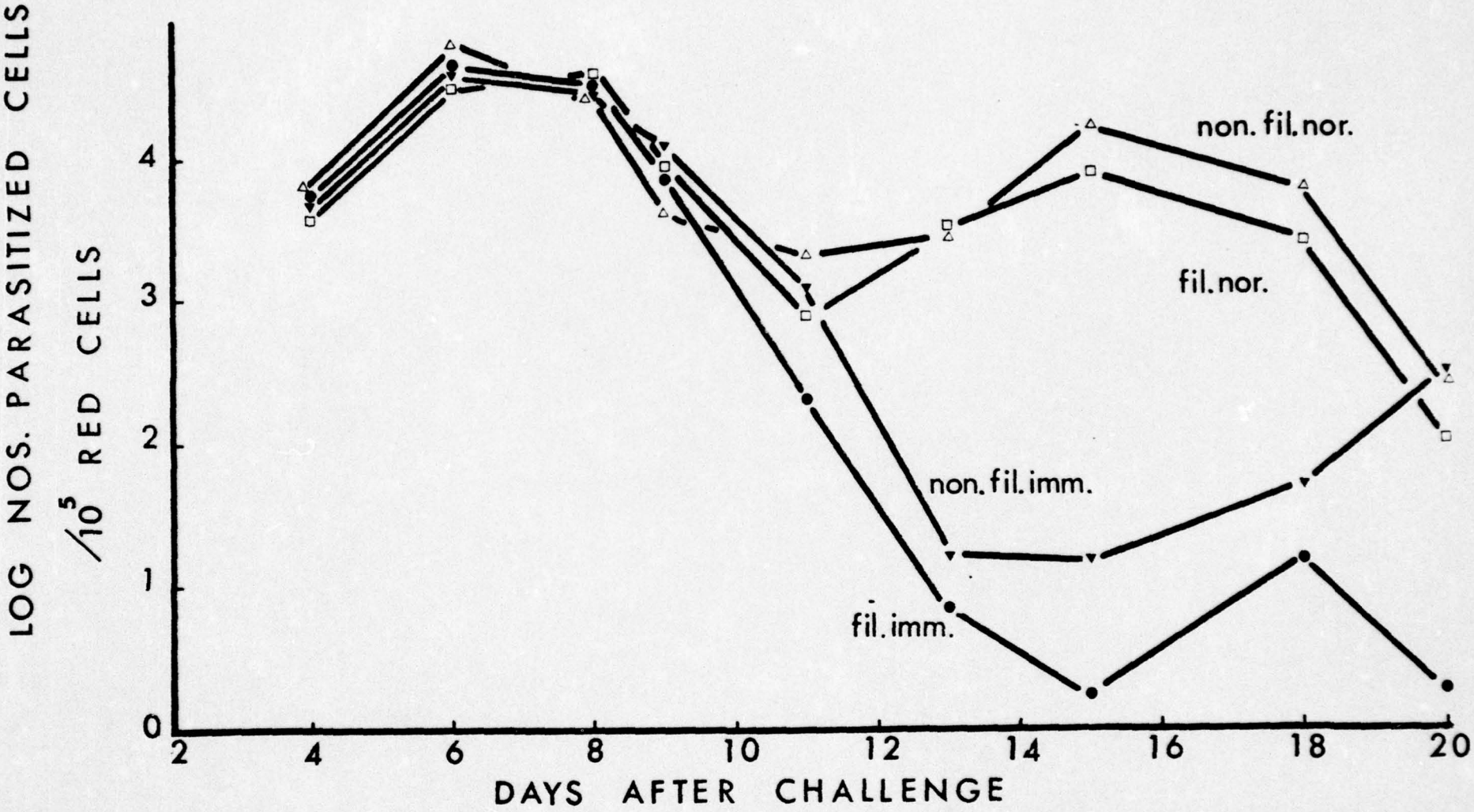


Fig. 9. Immunity transferred with immune spleen cells passed through glass-wool columns. (●—●) glass-wool filtered immune cells; (□—□) glass-wool filtered normal spleen cells; (▼—▼) non-filtered immune cells and (△—△) non-filtered normal cells.

Bone-marrow cells were flushed from the femur and tibia with medium 199 containing heparin.

The recipient mice were usually irradiated with a Siemens II X-ray machine and unless otherwise stated they were supplied with drinking water containing terramycin (3 g/l).

A number of different types of experiments have been carried out. Representative experiments will be described.

1. Comparison of protection after spleen cells injected i.v. or i.p.

In the majority of experiments to be described below relatively small numbers of immune or normal spleen cells were transferred. Early experiments showed that it was essential for these small numbers of cells to be injected intravenously rather than intraperitoneally in order for them to give some degree of protection to the recipients. For example, in the experiment shown in Figure 8, non-irradiated C57Bl mice received 9×10^6 immune or normal spleen cells (the spleen cells were passed through the glass-wool column but not through the nylon wool) either i.v. or i.p. and they were challenged with 1×10^6 P. chabaudi parasitized red cells i.p. one hour after cell transfer. It can be seen that little or no immunity was conferred on the immune cell recipients (six mice per group) injected i.p. whereas mice receiving the same cells i.v. had a significantly shorter patent primary parasitaemia compared with the controls.

Similar results were obtained with the transfer of immunity to P. berghei in CBA mice with immune spleen cells. In this case, 10^8 spleen cells were transferred and although some degree of immunity was transferred with the cells given both i.p. and i.v., the immunity conferred was significantly greater where the cells were injected intravenously.

2. Comparison of immunity transferred with different cell populations

(a) Cells passed through glass-wool column only

Glass-wool filtration of spleen cells removes clumps of cells, dead cells and many of the macrophages. In two experiments the immunity transferred with immune spleen cells before and after filtration through a glass-wool column was compared. In one experiment (Figure 9), non-irradiated male C57Bl mice, 5 months old, received 9×10^6 glass-wool column filtered, or non-filtered spleen cells i.v. from P. chabaudi immune or normal mice. The immune and normal mice were approximately nine months old when killed and the immune mice had been infected with P. chabaudi ninety days before sacrifice. The cell recipients, six mice per group, were challenged i.p. with 10^6 P. chabaudi parasitized cells one hour after cell transfer. The geometrical mean parasitaemias, given in Figure 9, show that in both immune cell recipient groups the primary patent parasitaemia was shortened compared with the non-immune spleen cell recipients. One mouse died and that in the filtered immune spleen cell recipients. That apart, there was a suggestion that the relapse parasitaemias were of longer duration and reached a higher peak in the recipients of the non-filtered immune cells.

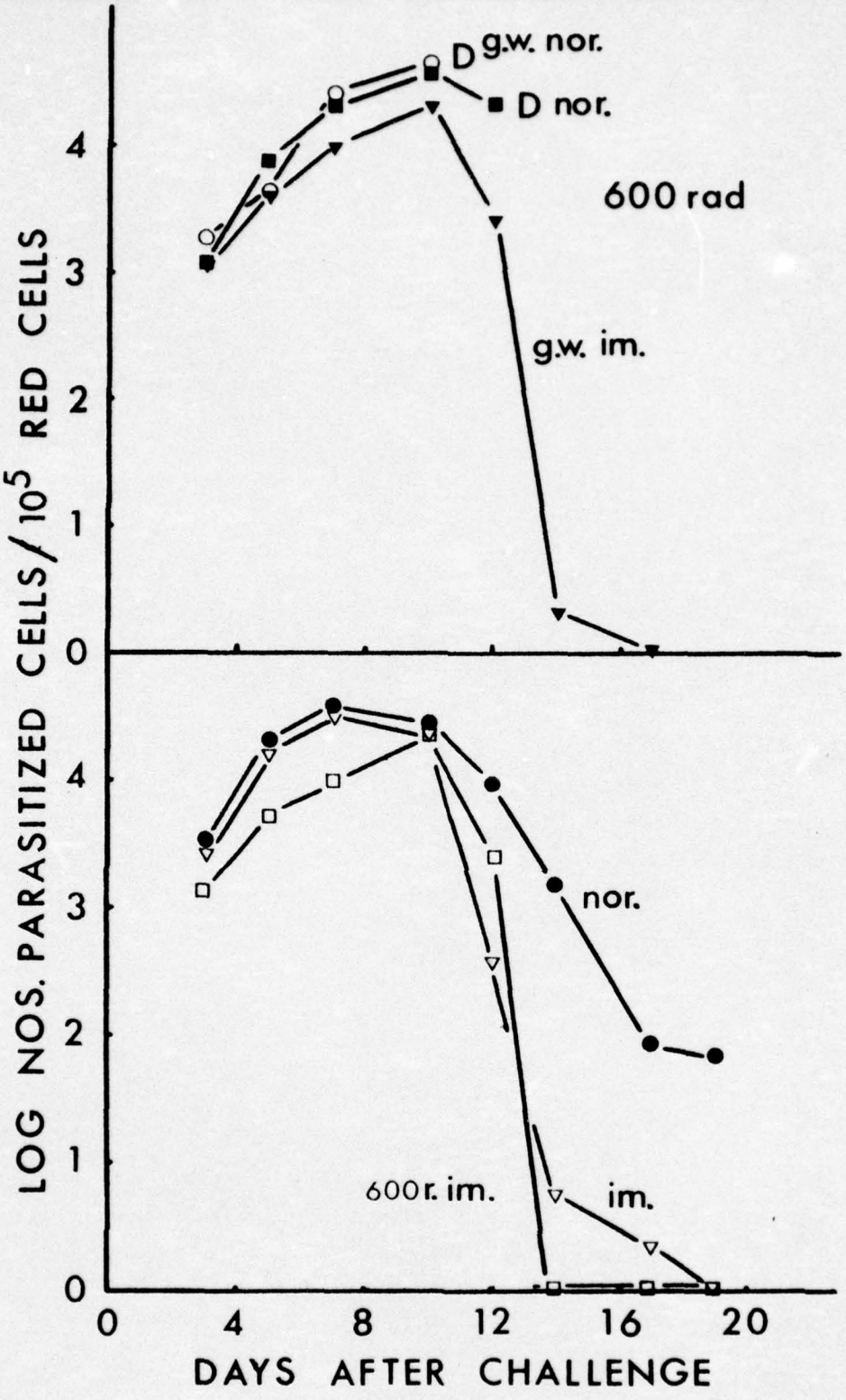


Fig. 9A. Glass-wool filtered immune spleen cells: (O——O) glass-wool filtered (g.w.) normal cells-irradiated recipients; (■——■) non-filtered normal cells - irradiated recipients; (▼——▼) g.w. immune cells - irradiated recipients; (□——□) non-filtered immune cells - irradiated recipients; (●——●) non-filtered normal cells - non-irradiated recipients; (▽——▽) non-filtered immune cells - non-irradiated recipients.

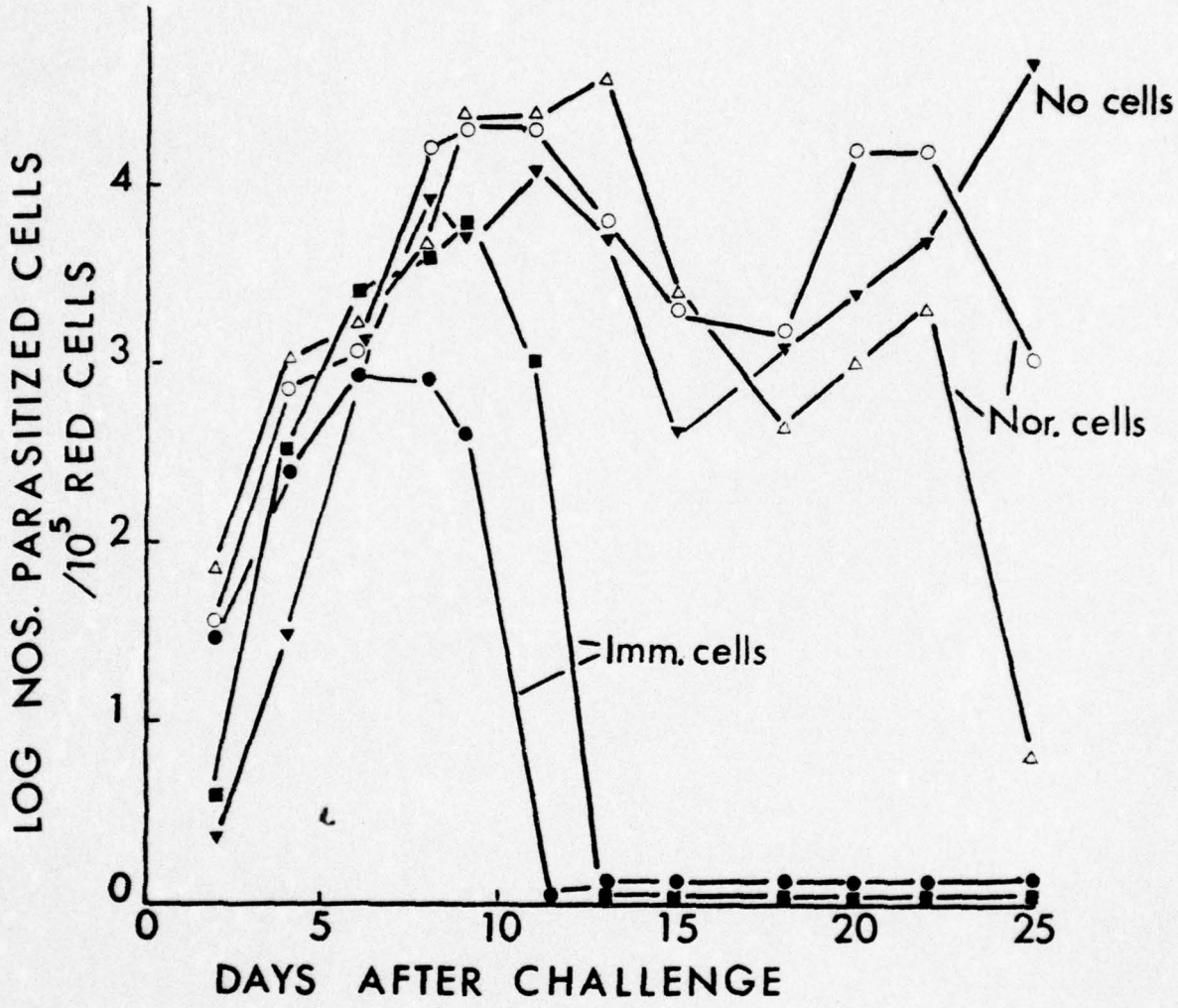


Fig. 10. Transfer of different numbers of immune cells. (●——●) 1.4×10^7 and (■——■) 7×10^6 immune spleen cells; (△——△) 1.4×10^7 and (○——○) 7×10^6 normal spleen cells; (▼——▼) irradiated recipients receiving no cells.

In a second experiment, CBA mice were used and most of the cell recipients were given 600 rads X-irradiation one hour before cell transfer. The recipient mice and cell donors were approximately one year old males. The immune donors had been reinfected with P. chabaudi once, 100 days before cell transfer. Groups each of six irradiated mice were set up which received i.v. 8×10^6 spleen cells, either filtered or non-filtered cells from normal or immune donors. Two groups of non-irradiated recipients were set up, one receiving non-filtered immune cells and the other non-filtered normal cells. All the mice were challenged i.p. with 3×10^6 P. chabaudi parasitized cells the day following cell transfer. The geometrical mean parasitaemias are given in Figure 9A. All the irradiated mice given normal spleen cells died twelve to thirteen days after challenge with an acute malaria infection. All the filtered immune cell recipients survived the primary parasitaemia and five of the six irradiated mice given the non-filtered immune cells. In all the surviving immune cell recipients the primary parasitaemia became subpatent about the same time. In all the irradiated mice the early primary parasitaemias were lower than in the non-irradiated mice and in the immune cell recipients, the peak parasitaemia in the irradiated mice was below that of the non-irradiated immune cell recipients. This difference in the course and height of the parasitaemias in irradiated and non-irradiated mice is a consistent finding.

These two experiments show that filtration of immune spleen cells from mice which were last infected 90-100 days previously (and had almost certainly effected a sterilizing immunity) on glass-wool columns did not diminish the protective activity of the effluent cells in the recipients and there was an indication in the first experiment that it was enhanced. This latter observation needs further investigation but this result does not suggest an important role for immune macrophages themselves in immune mice about three months after infection. In chronically infected, i.e. partially immune mice, the loss of cells adhering to the glass-wool may lead to a diminution of the ability of the spleen cells to protect recipients.

3. Protection in relation to numbers of immune cells transferred

The protective effect of immune cells is seen in the cell recipients usually as a shortening and a depression of the primary and relapse (if they occur) patent parasitaemias and where deaths occur in the controls, as a lowering of the death rate in the immune cell recipients. The degree of protection reflects the numbers of immune cells transferred. This observation has been made many times and a single illustration is given.

Three male NIH mice, immune to P. chabaudi, and last challenged 100 days previously, provided the immune spleen cells which were passed through a glass wool column. Normal spleen cells came from nine-week old male NIH mice, and the recipients were of the same age and sex as these mice. The cell recipients received 600 rads irradiation one hour before cell transfer. Groups of mice were set up which received either 7×10^6, or 1.4×10^7 immune or normal spleen cells i.v. A further group of irradiated mice received no cells. All the recipient mice were immediately challenged with 2×10^6 P. chabaudi parasitized cells i.p. The geometrical mean parasitaemias for the four groups are shown in Figure 10. It can be seen that in the immune spleen cells the primary parasitaemias was shortened compared with the

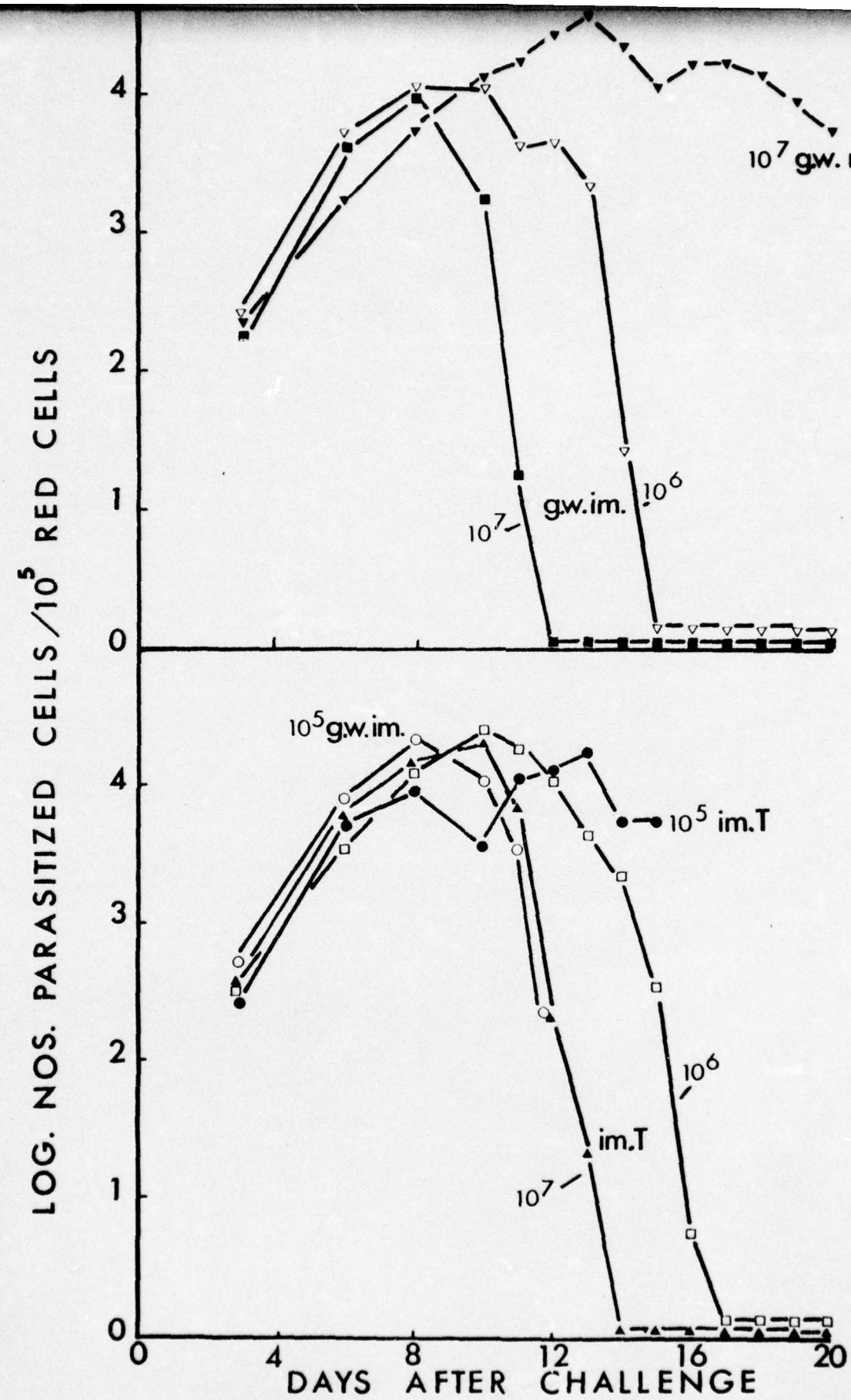


Fig. 11. Enriched T-cell populations. 10^7 normal cells - 2/6 deaths (▽——▽); 10^5 enriched T-cells - 6/6 deaths (●——●); 10^6 enriched T-cells - 2/6 deaths (□——□); 10^7 enriched T-cells - 1/6 deaths (▲——▲); 10^5 glass-wool filtered (g.w.) immune cells - 6/6 deaths (○——○); 10^6 g.w. immune cells - 2/6 deaths (▽——▽); 10^7 g.w. immune cells - 1/6 deaths

normal spleen cell recipients and the mice which received no cells and that doubling the number of immune spleen cells shortened the patent primary parasitaemia by approximately two days. It was also noticeable that the irradiated mice injected with normal spleen cells controlled their parasitaemias more quickly than the mice not injected with normal spleen cells.

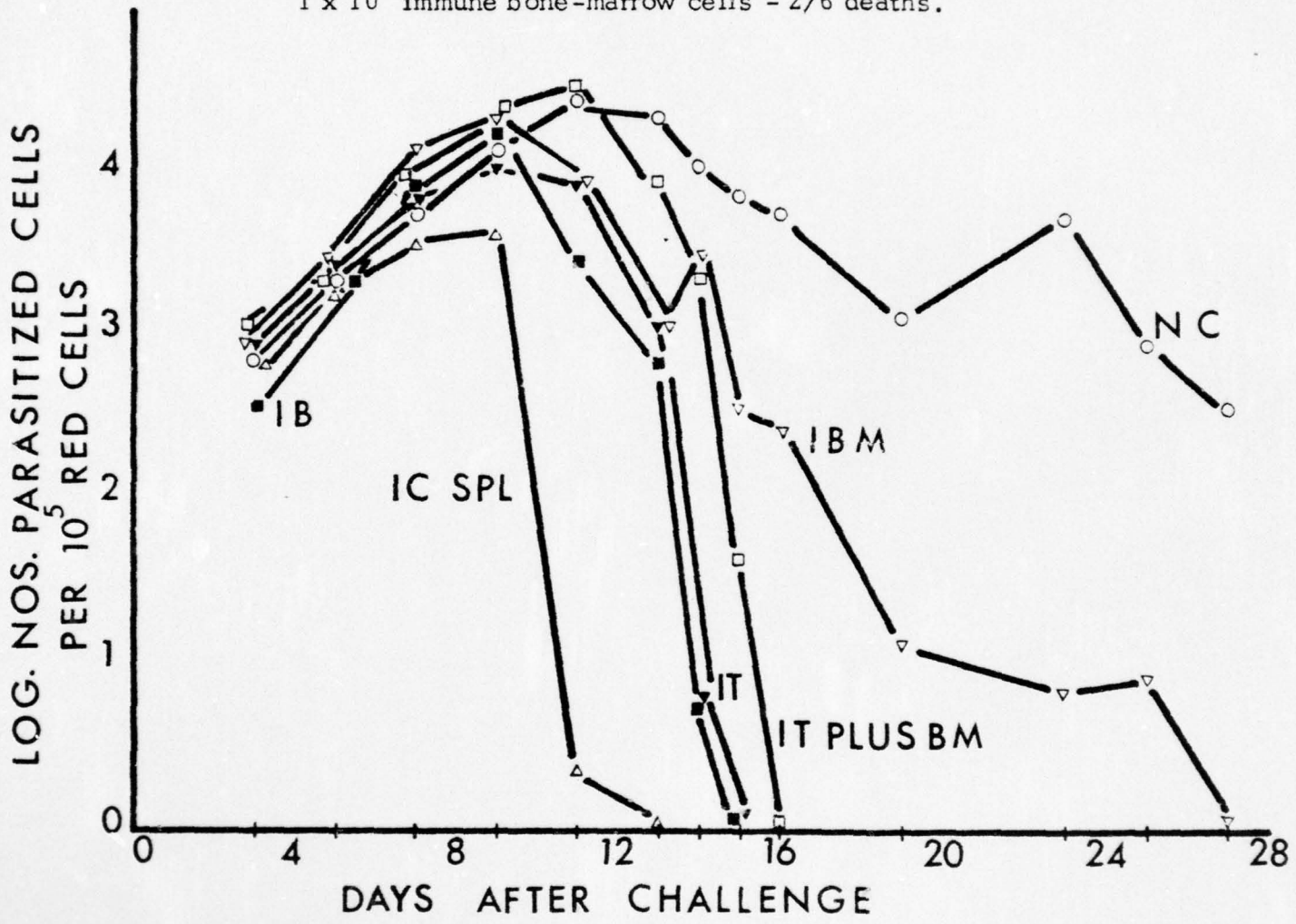
4. Immunity transferred with immune spleen cells after glass-wool and nylon-wool filtration

Fifteen experiments have been carried out to date and selected experiments are first described to illustrate those findings which have been consistently obtained. At the end of this section a few experiments will be described which have interesting, if unexplained and as yet unconfirmed, findings.

1. Immunity transferred with enriched T-cell populations

Immunity could usually be transferred with enriched T-cell populations. The following experiment illustrates this. The cell recipients were female NIH mice, approximately twenty weeks old, and they were irradiated with 800 rads X-irradiation two hours before cell transfer. The immune spleen cells were pooled from three female NIH mice, eighteen months old, first infected with P. chabaudi at six weeks and reinfected for the second time 140 days before cell transfer. Groups of six recipient mice were set up which received either 10^5, 10^6 or 10^7 of the enriched immune T-cell population (immune T-cells), or 10^5, 10^6 or 10^7 of the immune spleen cells subjected to glass-wool filtration only. A control group of mice received 10^7 normal spleen cells filtered through a glass-wool column. All the cells were injected i.v. and all the recipient mice were challenged with 2×10^6 P. chabaudi parasitized cells thirty minutes after cell transfer. The percentage of the cells in each population which became labelled with fluorescein-tagged anti-Ig was 22% and 29.5% for the glass-wool filtered immune and normal spleen cells respectively and 3% for the immune T-cell population. Geometrical mean parasitaemias after challenge are given in Figure 11. The following points are made. Mice receiving 10^7 immune cells were protected in that the primary patent parasitaemia was considerably shorter than in the normal spleen cell recipients. The patent parasitaemia in the 10^7 immune T-cell recipients was extended by about two days beyond that in the mice given 10^7 glass-wool filtered immune cells. For the 10^6 immune cell recipients the same pattern was found in the surviving mice: the immune cells shortened the patent parasitaemia compared with the control mice and in the enriched T-cell recipients patency was prolonged by one to two days beyond that in the glass-wool filtered immune cells. There was severe mortality in the mice given 10^5 immune cells, a consequence in part of the high dose of irradiation with inadequate stem-cell replacement.

Fig. 12. Enriched B-cell populations. (○——○) Group 1 - 2×10^6 glass-wool filtered (g.w.) normal cells - 1/6 deaths; (△——△) Group 2 - 2×10^6 g.w. immune cells - 0/6 deaths; (■——■) Group 3 - 2×10^6 enriched immune B-cells - 0/6 deaths; (▼——▼) Group 4 - 2×10^6 enriched immune T-cells - 1/6 deaths; (▽——▽) Group 5 - 2×10^6 immune bone-marrow cells - 3/6 deaths; (□——□) Group 6 - 1×10^6 enriched immune T-cells + 1×10^6 immune bone-marrow cells - 2/6 deaths.



ii. Immunity transferred with enriched B-cell populations

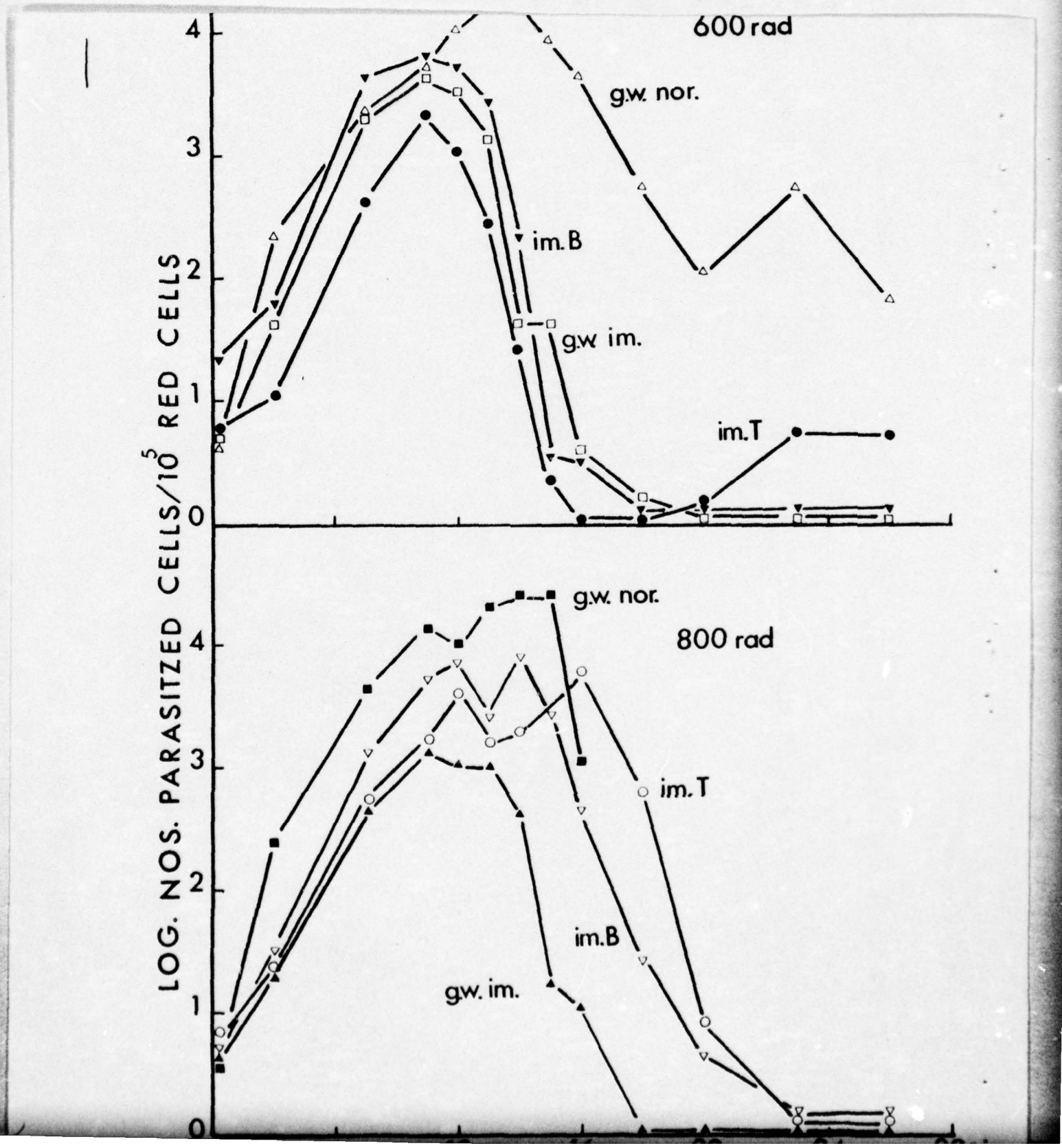
Th enriched B-cell population was obtained by dislodging some of the cells, which had attached to the nylon-wool column, by pushing medium through the column with the syringe plunger.

Immunity could be transferred with enriched immune B-cells but the level of immunity associated with this population of cells relative to a enriched T-cell population or unfractionated cells tended to vary. A representative experiment is described.

The recipient mice were twelve-week old female mice, irradiated with 800 rads two hours before cell transfer. The immune spleen cells were pooled from two female NIH mice seven months old, first infected with P. chabaudi when six weeks old and reinfected sixteen days before cell transfer. The normal spleen cells came from two five-month old female NIH mice. Six groups of recipients were set up with six mice in each group as follows: Group 1 - 2×10^6 normal (glass-wool filtered) spleen cells, Group 2 - 2×10^6 immune (glass-wool filtered) spleen cells, Group 3 - 2×10^6 enriched immune B-cell population, Group 4 - 2×10^6 enriched immune T-cell population, Group 5 - 2×10^6 immune bone-marrow cells and Group 6 - 1×10^6 enriched immune T-cells and 1×10^6 immune bone-marrow cells. The percentage of cells labelled with the fluoroscein-tagged anti-mouse Ig was 57% for the enriched B-cells, 2% for the enriched T- and 26% for the glass-wool filtered immune spleen cells. All the recipient mice were challenged with 2×10^6 P. chabaudi parasitized cells thirty min after cell transfer was completed and the geometrical mean parasitaemias for each group after challenge is given in Figure 12.

It can be seen that all the immune cell recipients were protected to some degree in that their primary patent parasitaemias were shortened in comparison with the controls. The best protection was given by the immune spleen cells subjected to glass-wool filtration only. In the enriched T-cell and enriched B-cell recipients the patent parasitaemia lasted three to four days longer than in the glass-wool filtered immune cells. Substitution of 10^6 enriched immune T-cells by 10^6 immune bone-marrow in Group 6 prolonged the patent parasitaemia by about one day compared with that in the 2×10^6 enriched T-cell recipients (Group 4). Immune bone-marrow cells gave the poorest protection.

In other experiments the enriched B-cell population when transferred to 600 rad or 800 rad irradiated recipients gave in three experiments slightly better protection than the enriched T-cells, and in two others comparable protection. Apart from one experiment where the recipients received 600 rads before cell transfer and the pattern of the parasitaemia followed the same course in all immune cell recipients, the enriched B-cell and enriched T-cell population invariably gave a degree of protection to the recipients which was inferior to that provided by immune spleen cells subjected to glass-wool filtration alone.


600 rad
g.w. nor.
im.B
g.w im.
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800 rad
g.w. nor.
im.T
im.B
g.w. im.
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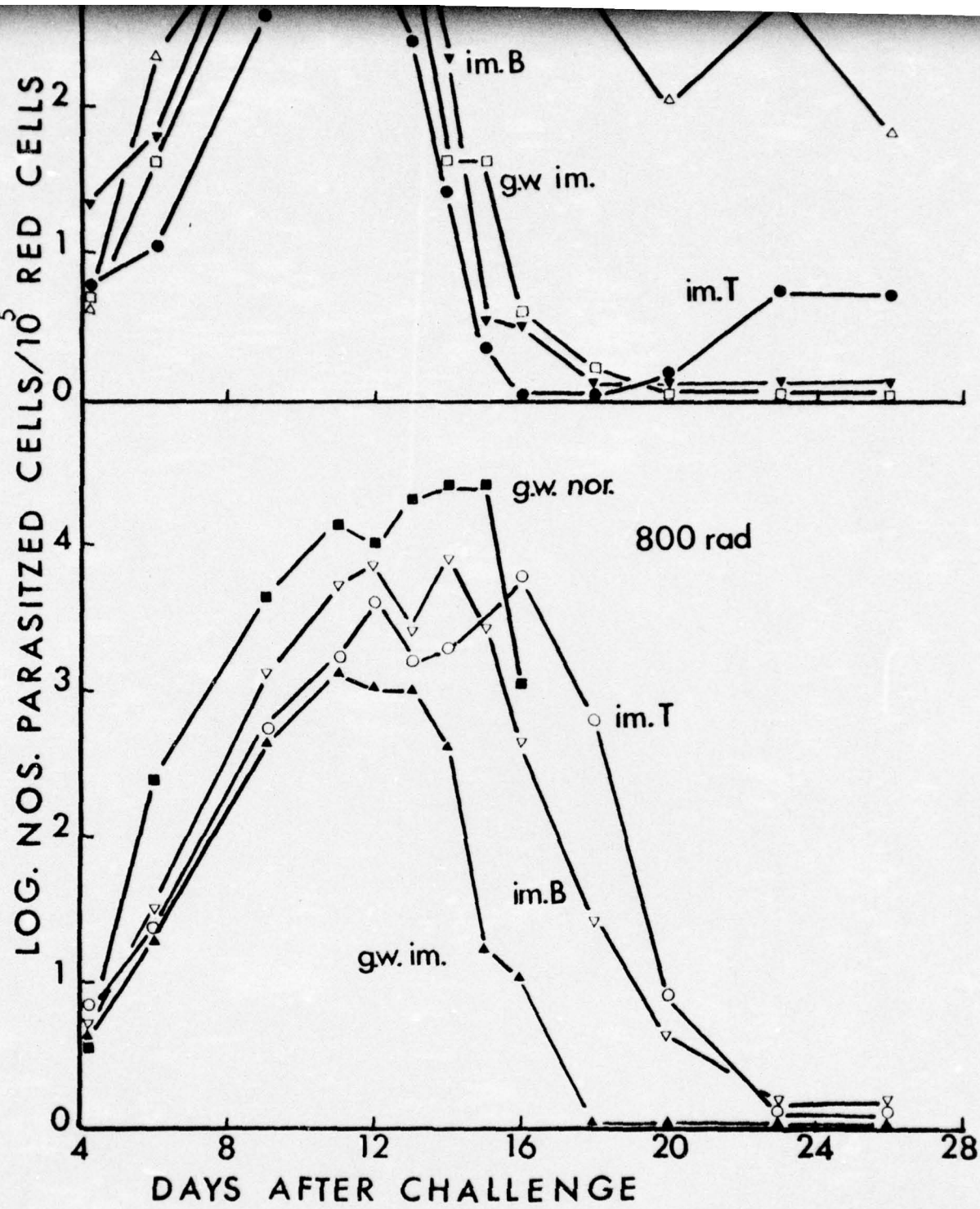


Fig. 13. Immune cells in 600 rad or 800 rad irradiated recipients. (○——○) immune T-cells, 800 rad recipients, 1/6 deaths; (▽——▽) immune B-cells, 800 rad recipients, 1/6 deaths; (▲——▲) glass-wool filtered (g.w.) immune cells, 800 rad recipients, 0/6 deaths; (■——■) g.w. normal cells, 800 rad recipients, 7/7 deaths; (●——●) immune T-cells, 600 rad recipients, 0/7 deaths; (▼——▼) immune B-cells, 600 rad recipients, 0/6 deaths; (□——□) g.w. immune cells, 600 rad recipients, 1/6 deaths; (△——△) g.w. normal cells; 600 rad recipients, 3/6 deaths.

iii. Comparison of protection provided by frationated and unfractionated immune spleen cells in recipients given 600 or 800 rads before cell transfer

It was shown that immunity could be transferred with an enriched T-cell population. One possible way in which these T-cells could mediate an anti-parasitic effect is by co-operation with B-cells in the recipient mice. After irradiation the B-cell numbers in mice rapidly fall (Mossal et al., 1974). After high doeses of irradiation few B-cells remain and if the irradiated mice are not reconstituted with stem cells, there is a slow replenishment of B-cells. Thus it was thought that if the anti-parasitic activity of the immune T-cells is regulated by the supply of B-cells then the enriched T-cell population could be more effective in mice given 600 rads than in 800 rad irradiated mice. In four experiments immune spleen cells were transferred to irradiated mice given 600 rads or 800 rads. A representative experiment is described and the important points from the others are discussed.

The recipients were female NIH mice, seventeen weeks old, and they received 800 or 600 rad irradiation two hours before cell transfer. The immune spleen cells were pooled from three female mice, twelve months old, immune to P. chabaudi, and last reinfected sixty-five days before cell transfer. Normal spleen cells came from two female NIH mice, seventeen weeks old. Eight groups of cell recipients were set up, four groups of 600 rad irradiated mice and four groups of 800 rad irradiated. The recipients received 10^6 spleen cells i.v. of either an enriched immune T-cell population (Groups 3 and 7), immune spleen cells subjected to glass-wool filtration only (Groups 2 and 6), or glass-wool filtered normal spleen cells. In the immune cells population the proportion of cells labelling with the anti-mouse Ig antibody was 1.3% of enriched T-cells, 52% of enriched B-cells and 24% of the glass-wool filtered cells. All the recipient mice were challenged with 10^5 P. chabaudi parasitized cells one hour after cell transfer was completed. The geometrical mean parastiaemias are given in Figure 13 which illustrates the following points. In the 800 rad recipients the highest parasitaemias were seen in the normal spleen cell recipients (all died), followed by the enriched B-cell population, and then the enriched T-cells and the lowest parasitaemias were in the glass-wool filtered immune cells. The primary parasitaemia in the enriched immune B-cells and enriched immune T-cell population characteristically became subpatent three to four days after the glass-wool filtered immune spleen cells. In the 600 rad recipients, the severest parasitaemias were again to be seen in the normal spleen cell recipients. The primary patent periods in all the immune cell recipients for the most part followed a similar course although the enriched T-cell and enriched B-cell recipients became subpatent shortly before the glass-wool filtered (G.W.) immune spleen cells. Comparing the same cells in 600 and 800 rad recipients we see that for the G.W. immune cells the parasitaemias followed a similar course but that the peak parasitaemias were higher in the 600 rad recipients. In these mice (G.W. immune cell recipients) up to day 48 after transfer and challenge one of five mice in the 600 rad recipients and 0 of six mice in the 800 rad recipients had relapsed. For the enriched T-cell recipients, the height of the parasitaemias in the 600 and 800 rad recipients was about the same but the duration of the primary patent parasitaemia was extended in the 800 rad recipients. Three of five mice in the

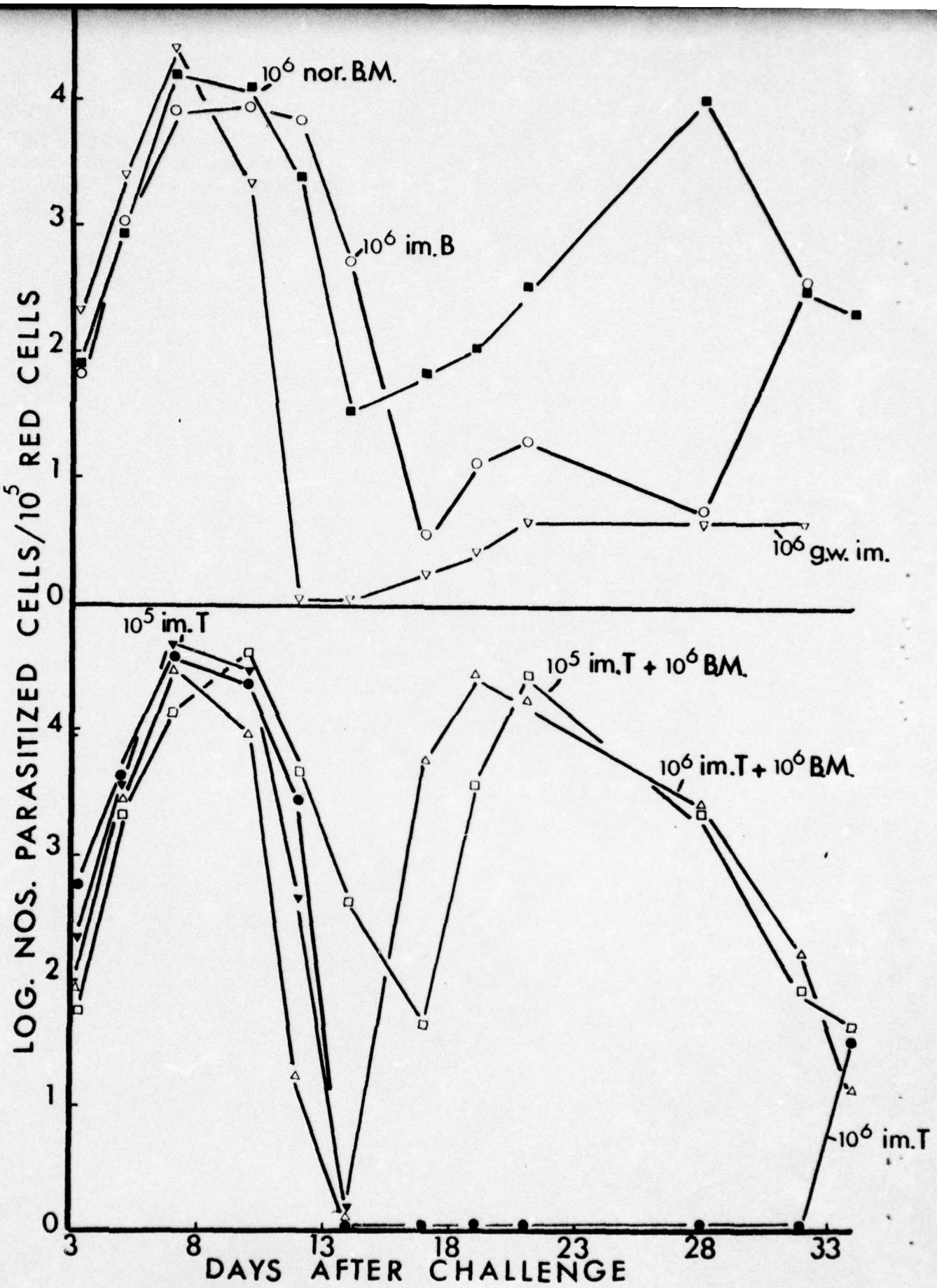


Fig. 14. Enriched T-cells in presence of bone-marrow cells. (▾——▾) 10^5 enriched immune T-cells; (●——●) 10^6 enriched immune T-cells; (○——○) 10^6 enriched B-cells; (▿——▿) 10^6 glass-wool filtered immune cells; (■——■) 10^6 normal bone-marrow cells; (□——□) 10^5 10^6 10^6 + 10^6

latter group had patent relapses and seven of seven in the 600 rad recipients up to day 48. In the enriched B-cell recipients, the parasitaemias were again extended in the 800 rad recipients: none of the enriched B-cell recipients showed a patent relapse parasitaemia. In this respect these mice behave like the G.W. immune cell recipients and were in contrast to the enriched T-cell recipients. Finally, all the normal spleen cell recipients at 800 rads died between days 12-18 of acute malaria. At 600 rads, three of six mice died between days 15 and 20 and the remaining mice had patent primary parasitaemias lasting for 26-31 days after challenge and all later relapsed.

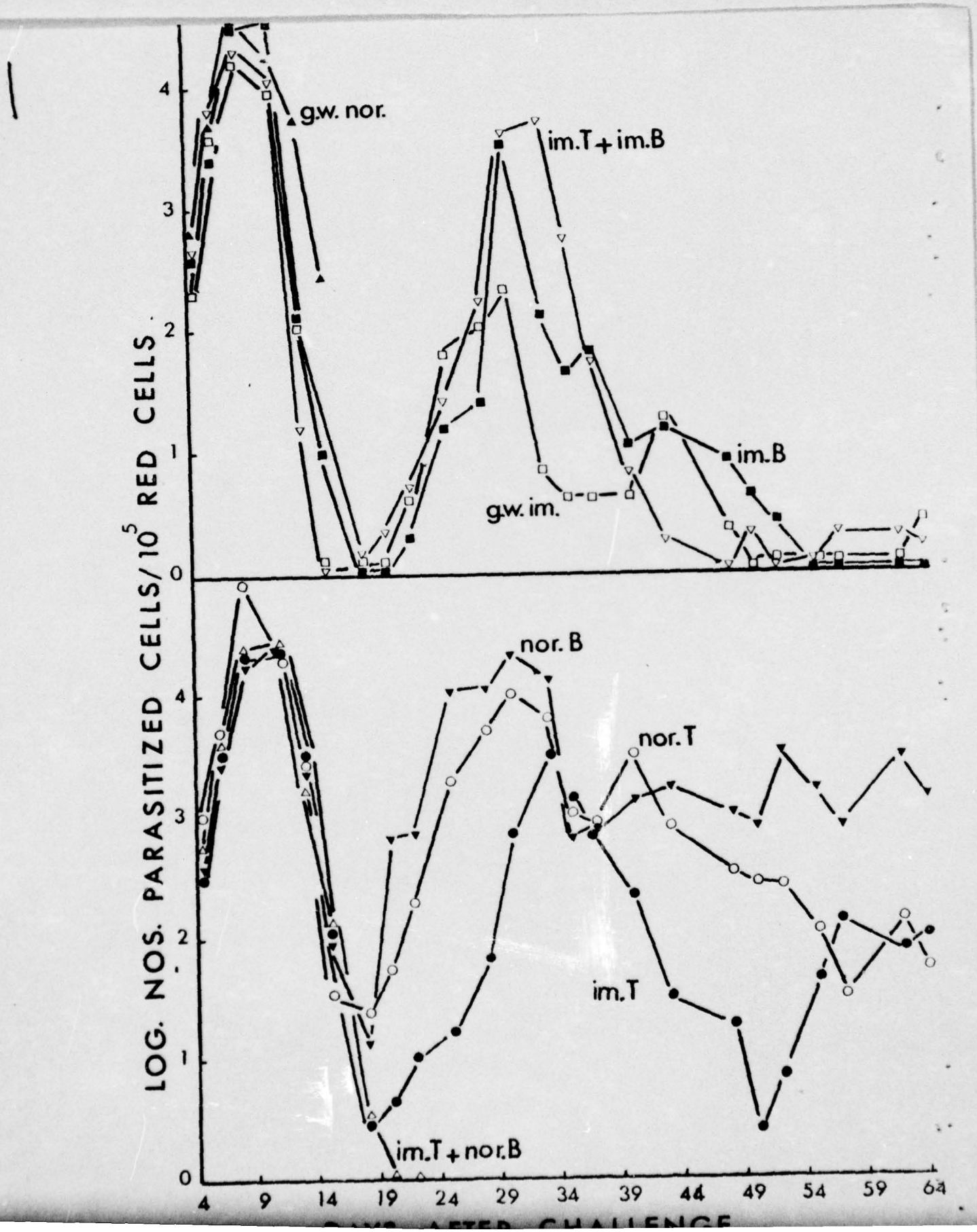
In this experiment, taking as the criteria for protection in the recipients as the height and duration of the primary patent parasitaemia after challenge and the occurrence of a patent relapse parasitaemia we can summarise the findings as follows. The enriched B- and enriched T-cell populations were less protective in the 800 rad recipients than the G.W. filtered immune spleen cells. Overall the enriched T-cell population was least protective although in the 600 rad recipients this was only based on the occurrence of relapse parasitaemias in the recipients. It has to be noted that at the lower irradiation dose there was clearly some contribution by the recovering immune system of these mice.

Other experiments have confirmed these observations comparing protection in mice given varying doses of irradiation with one exception in which the enriched immune T-cell population conferred approximately the same degree of protection on 600 and 800 rad irradiated mice.

iv. Addition of bone-marrow or enriched B-cells to enriched T-cell recipients

Enriched immune T-cell populations had been found to be protective in irradiated recipients, albeit in the majority of experiments with lethally irradiated recipients, less protective than glass-wool filtered immune spleen cells. As indicated above the protection derived from the T-cells may be dependent on viable B-cells for their activity, i.e. through the cooperation of T- and B-cells in the production of protective antibodies. A number of experiments have been carried out in which the T-cell recipients also received bone-marrow cells or an enriched B-cell population. For a number of reasons, e.g. an unexpected number of deaths from irradiation, the results have not been conclusive but nevertheless they may be indicative of what is taking place.

In the first experiment male C57Bl mice were used. The immune mice had been reinfected 55 days before cell transfer. The recipients, seven weeks old, were given 600 rad irradiation two days before cell transfer. Groups of five or six recipient mice were set up and they received cells i.p. as shown in Figure 14. The cells were transferred two days after the mice were irradiated and all the recipient mice were challenged with 5×10^6 P. chabaudi parasitized cells two hours after cell transfer was completed. There were deaths in all the groups except Group 7 (glass-wool filtered immune spleen cells). Comparing the geometrical mean parasitaemias for the surviving mice it can be seen that (judged by the criteria of the length of the primary patent parasitaemia and the interval between the end of the primary patent parasitaemia and a relapse parasitaemia), Group 7 were best protected followed by Group 1 (10^6 immune T-cells) and Group 6 (10^6 immune B-cells). The interesting


g.w. nor.
im.T + im.B
im.B
g.w. im.
nor. B
nor.T
im.T
im.T + nor.B
LOG. NOS. PARASITIZED CELLS/ 10^5 RED CELLS
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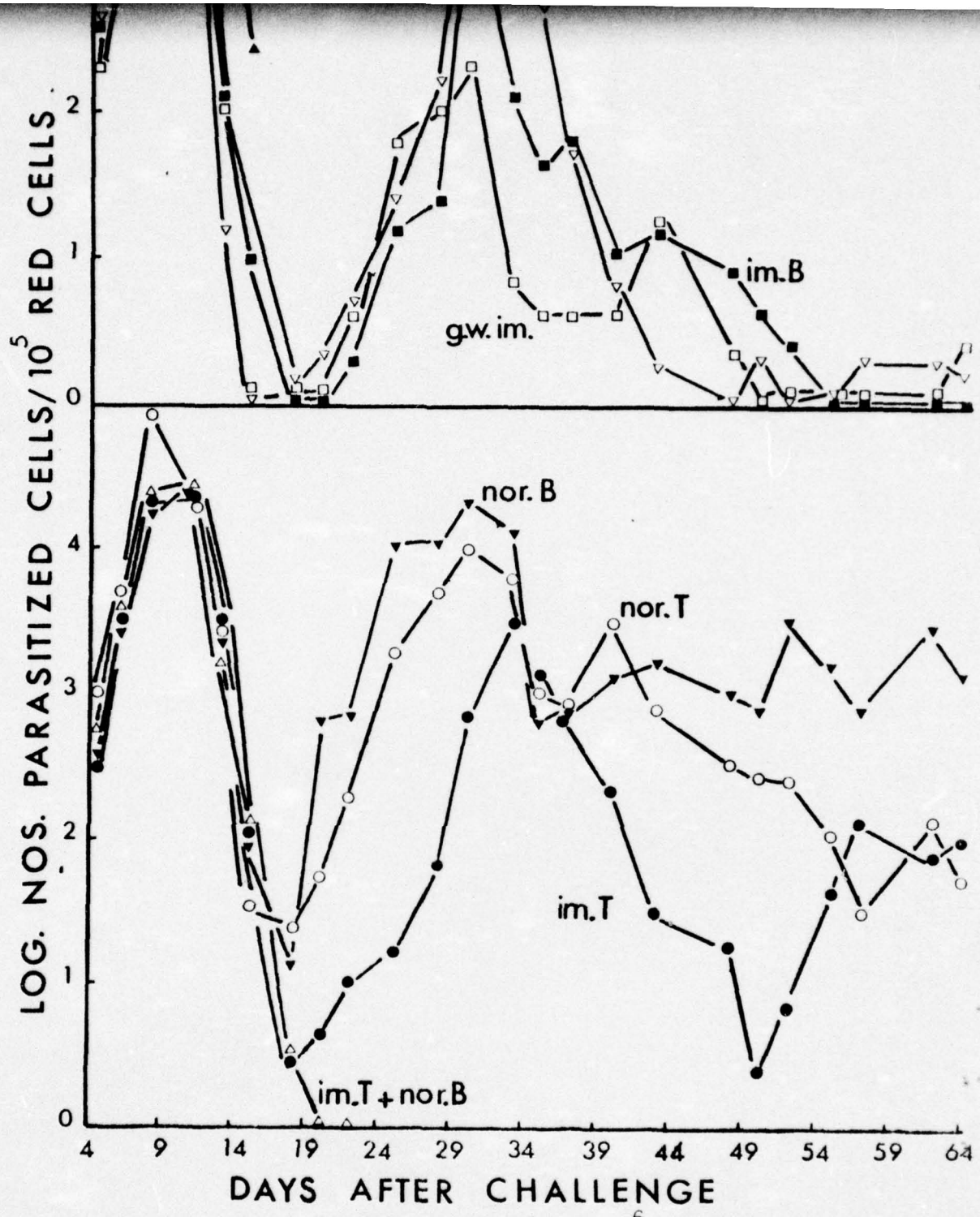

Fig. 15. Enriched T-cells in presence of B-cells. (●——●) 10^6 enriched immune T-cells - 2/6 deaths; (□——□) 10^6 glass-wool filtered (g.w.) immune cells 0/6 deaths; (■——■) 10^6 enriched immune B-cells - 3/6 deaths; (○——○) 10^6 normal enriched T-cells; (▼——▼) 10^6 enriched normal B-cells; (▲——▲) 10^6 g.w. normal cells; (△——△) 10^6 enriched immune T + 10^6 enriched normal B-cells; (▽——▽) 10^6 enriched immune T + 10^6 enriched immune B-cells.

observation was that the addition of 10^6 normal bone-marrow cells to 10^6 immune T-cells (Group 2) rapidly precipitated acute relapse parasitaemias.

In another experiment with female NIH mice, the bone-marrow cells were taken from the immune spleen cell donors. The immune mice were reinfected sixteen days before cell transfer. The recipient mice, six months old, were irradiated with 800 rads approximately one hour before cell transfer. Groups of six mice received the spleen or bone-marrow cells i.v. as shown earlier in Figure 12 and the mice were challenged with 2×10^6 parasitized red cells i.p. immediately after cell transfer. Of relevance to the present discussion is that immune bone-marrow themselves gave a marginal level of protection and that there was no evidence that immune bone-marrow potentiated the activity of the enriched T-cell population.

Experiments in which irradiated mice received either mixtures of enriched immune or non-immune B- and enriched T- immune spleen cells or enriched T- or B-cells alone again did not show that the T-cells in the presence of B-cells had enhanced protective activity. One experiment is described to illustrate this. Female C57Bl mice were used. The immune donors were reinfected twenty-one days before cell transfer. The recipients, five months old, received 600 rads 24 hours before cell transfer. Eight groups of five or six mice were set up as shown in Figure 15. Spleen cells and the challenge inoculum of 10^6 parasitized cells were injected i.p., the parasitized cells being given the day after cell transfer. The geometrical mean parasitaemias and the deaths are given in the Figure. Unfortunately there were a number of deaths in some groups, deaths which could not be ascribed to the direct effects of the parasite. Nevertheless the results show that although the mixture of enriched immune T- and B- immune cells gave very slightly better protection than the immune T-cells and was comparable to that given by the glass-wool filtered immune spleen cells, this could be attributed to the increased number of cells given (2×10^6 cells in total) and not to any enhanced T-cell activity mediated through the B-cell population.

v. Cross-immunity between P. chabaudi and Babesia microti and B. hylomysci

In general there is no cross-immunity between strains of malaria parasites. The rodent malarias are unusual that there is some degree of cross-immunity between species, e.g. mice immune to P. berghei show resistance to P. chabaudi (see review Cox, 1975), although the reverse is not true. Even more surprisingly there has been found to be cross-immunity between rodent malarias and rodent babesias although the basis of this immunity has not been investigated. If this cross-immunity is mediated through protective antibody, it could be that sensitized T-cells from a mouse immune to malaria, for example, by recognising common antigens between the malaria parasites and the babesias can subsequently act as helper cells in the production of specific anti-babesia antibody when the immune mouse is infected with piroplasms. In this model we can look on the common antigens as the carrier and the specific malarial or babesia antigens, against which the protective antibodies are directed as the hapten (Brown, 1971; Brown & Phillips, 1971).

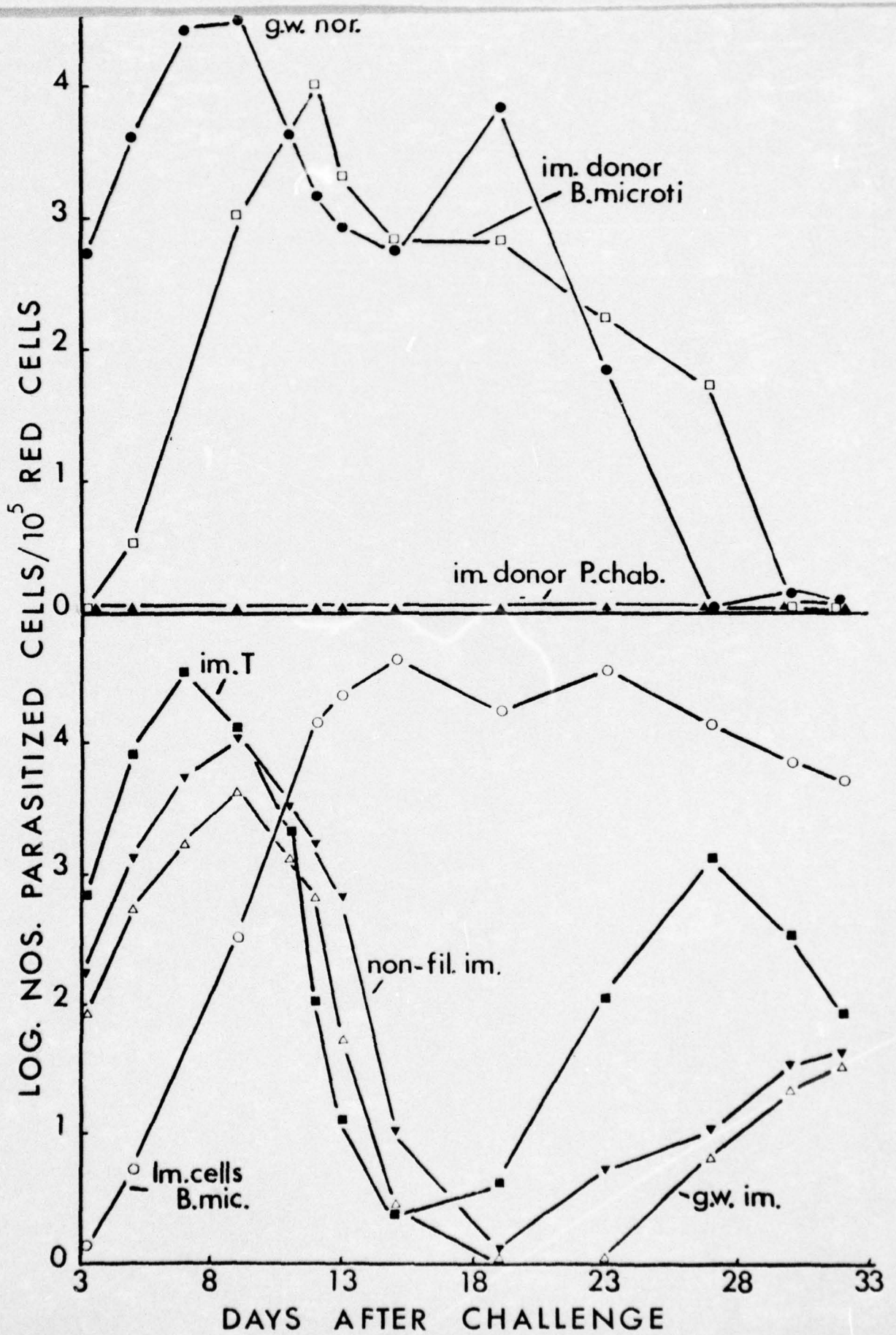

Fig. 16. Attempts to transfer cross-immunity with spleen cells. (o—o) all immune and

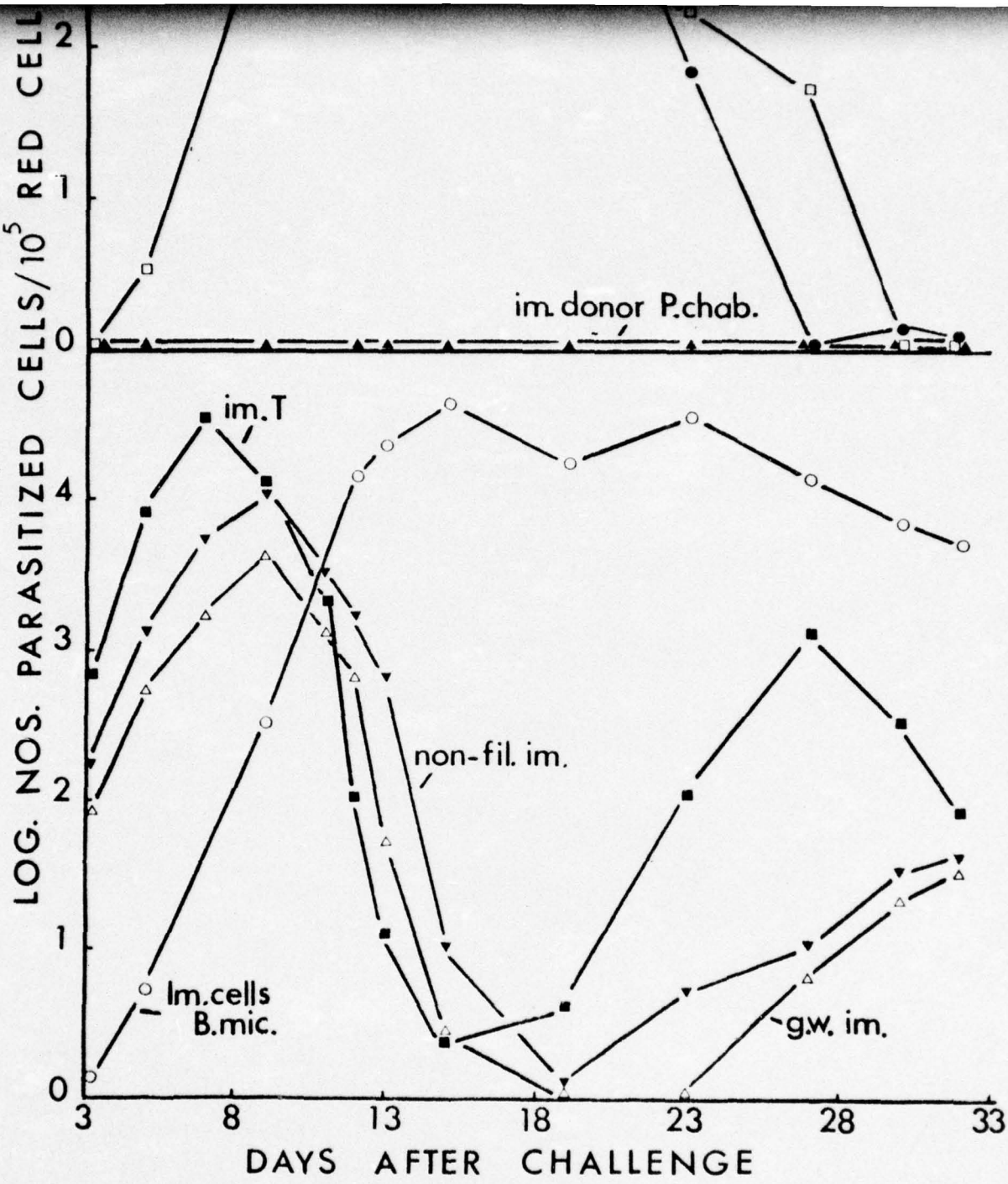

Fig. 16. Attempts to transfer cross-immunity with spleen cells. (○——○) all immune and normal cell recipients challenged with B. microti; (■——■) enriched immune T-cells - P. chabaudi; (△——△) glass-wool filtered (g.w.) immune cells - P. chabaudi; (▼——▼) immune spleen cells - P. chabaudi; (●——●) g.w. normal cells - P. chabaudi; (▲——▲) immune donors P. chabaudi: (□——□) immune donors - B. microti.

In the course of this study on the role of different lymphoid cells in malarial immunity three experiments have been carried out examining cross-immunity between P. chabaudi and Babesia hylomysci and B. microti. Although it was established that mice which had been reinfected with P. chabaudi 39 days previously, showed strong resistance to a B. hylomysci challenge, subsequently experiments showed that C57Bl mice which had last been reinfected with P. chabaudi 73 or 90 days before being challenged with B. hylomysci showed little or no resistance to the latter parasite but did to P. chabaudi. Spleen cell transfer (3×10^6 or 9×10^6 per mouse) from those mice immune to P. chabaudi gave no protection to non-immune recipients to a B. hylomysci challenge but did to a P. chabaudi challenge. In a third experiment the C57Bl mice immune to P. chabaudi and recipients of their spleen cells were challenged with B. microti, a less virulent species of rodent babesia. The immune mice, male C57Bl mice, had been infected with P. chabaudi 120 and 18 days before cell transfer, and 10^7 spleen cells were injected i.v. into each twelve-week old male C57Bl mouse. Non-immune spleen cells came from twelve-week old male mice. Pairs of groups of five or six mice were set up which received either glass-wool filtered normal or immune spleen cells, an enriched immune T-cell population, or a whole immune spleen cell population. The mice of one member of each pair of groups was challenged with 10^5 P. chabaudi parasitized cells i.v. and the other with 10^5 B. microti parasitized cells i.v. the day after cell transfer. Representative immune cell donors were also challenged with either P. chabaudi or B. microti. The geometrical mean parasitaemias for the cell recipients and the immune cell donors after challenge are given in Figure 16. It can be seen that the immune cell donors were as expected strongly resistant to P. chabaudi, and a measure of resistance was shown to B. microti. This was evident as a shortened patent parasitaemia and a lower peak parasitaemia compared with the cell recipients challenged with the same parasite. It was noticeable that there was no significant delay in the onset of the patent parasitaemia in the immune cell donors challenged with B. microti compared with controls (e.g. Group 8) which suggests that the cross-immunity may not have protective antibody as the major component. All the immune spleen cell recipients were provided with some degree of protection to P. chabaudi but during the 29 days after challenge none of the immune cell recipients challenged with B. microti showed any resistance.

5. A note on the effect of irradiation on the activity of passively transferred immune serum

It was noted in the previous Annual Report and elsewhere in this report that in heavily irradiated mice there was an apparently non-specific resistance in the early course of a P. chabaudi infection. In three experiments it has been shown that the anti-parasitic activity of immune serum can be amplified in irradiated mice. A representative result is described. Three-month old male C57Bl mice were used. The irradiated mice received 800 rads the day before serum transfer and infection. All the mice, irradiated and non-irradiated, were infected with 5×10^4 P. chabaudi parasitized cells i.v. and immediately afterwards received either 2.0 ml immune or normal serum i.p. The geometrical mean parasitaemia for each group is given in Figure 17 where it can

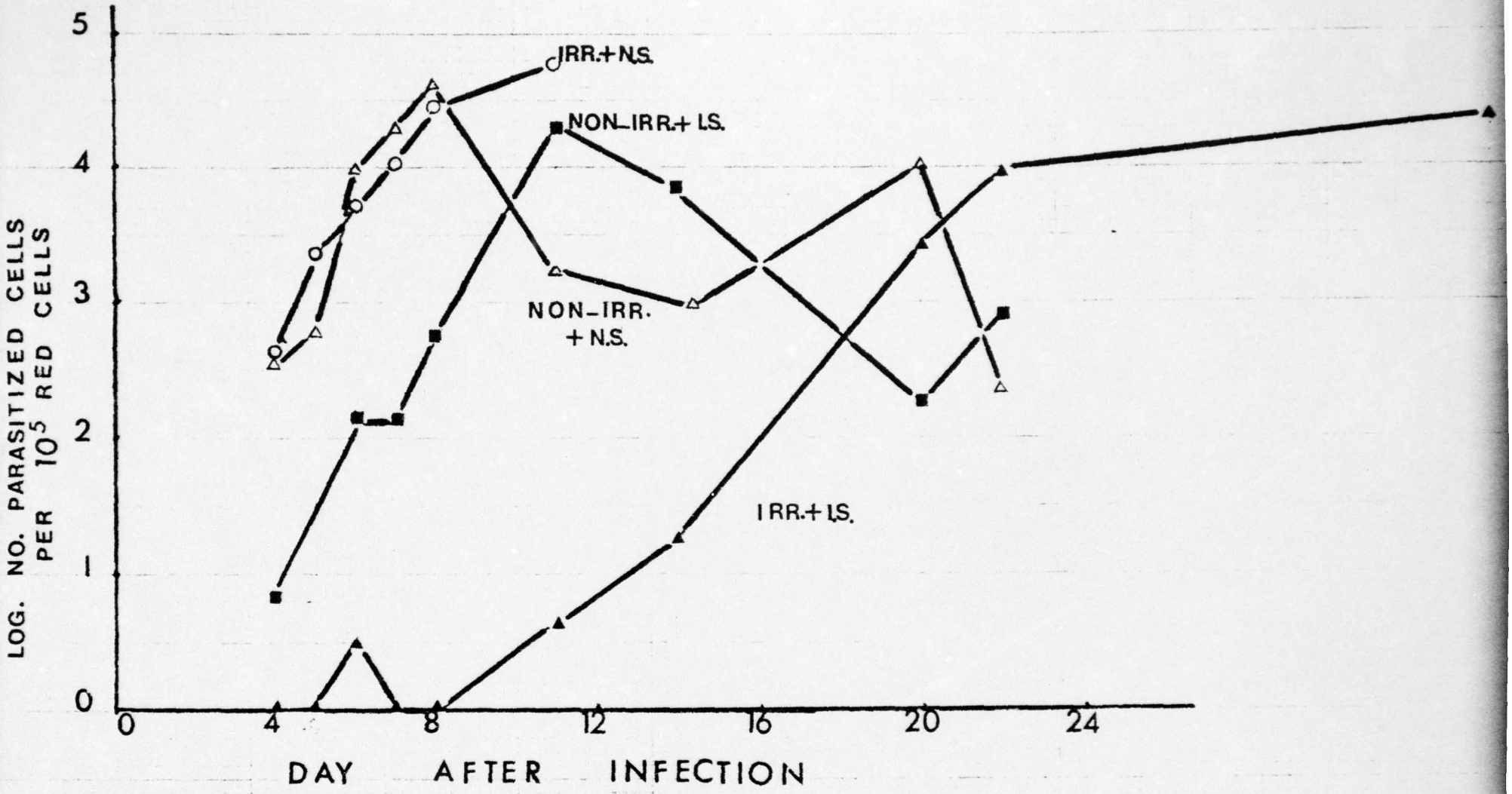

Fig. 17. Serum transfer in irradiated mice. (O—O) irradiated mice + normal serum; (▲—▲) irradiated mice + immune serum; (△—△) non-irradiated mice + normal serum; (■—■) non-irradiated mice + immune serum.

clearly be seen that the difference in the time of onset of the ascending primary parasitaemias between normal and immune serum recipients was clearly greater in the irradiated mice. An unusual feature of this particular experiment was that in the irradiated mice given normal serum, the onset and the rise of the patent parasitaemia occurred at the same time and at the same rate respectively as in their non-irradiated counterparts: in almost all experiments with 600 or 800 rad irradiated mice where non-irradiated mice were included for comparison, the rate of increase was depressed in the early phase of the patent parasitaemia in the irradiated mice.

Discussion

Mice infected with P. chabaudi experience an acute primary parasitaemia lasting 10-14 days and thereafter carry a chronic and usually sub-patent infection for 40 or 50 days or longer before completely eradicating the parasite. The acute parasitaemic episode followed by a period of chronicity is a feature of many malaria parasites in their hosts, including the malaria parasites of man. In a few species of malaria repeated antigenic variation is known to occur during the course of the infection; in P. chabaudi it is not yet certain that antigenic variation occurs although indirect evidence indicates that it does. In common with many other malaria parasites immunity to P. chabaudi can be transferred with serum. Phagocytosis of trophozoites, schizonts and possibly merozoites can be observed in immune mice given a large challenge infection and in mice in which the primary parasitaemia is regressing. Certainly loss of parasitized red cells from the peripheral circulation in immune or semi-immune mice occurs between the trophozoite stage and reinvasion. Protective sera therefore probably contain opsonizing antibody. At peak parasitaemia synchrony is lost to some extent, and abnormal looking forms, especially incompletely developed schizonts can be seen. It may be that some of the phagocytosed parasites or parasitized red cells are dead before being taken up by the phagocytic cells, killed by other immune processes. Parasites in which growth is apparently abnormal are probably damaged when the merozoites are released into the plasma prior to reinvasion. Clark et al. (1975) suggest that the 'crisis' forms are the result of the activity of non-antibody mediators for the release of which T-cells are necessary although the forms that Clark et al. describe as crisis forms are not necessarily similar to those observed by ourselves. Butcher & Cohen (1970) describe the activity of anti-merozoite antibody 'in vitro' in sera from rhesus monkeys immune to P. knowlesi which prevents free merozoites from invading red cells by apparently first agglutinating and then lysing them. Unfortunately P. chabaudi does not readily undergo reinvasion in vitro and hence the demonstration of an anti-P. chabaudi merozoite antibody remains to be made. In summary it was known that immunity to the blood stage of P. chabaudi in mice involved protective antibody. The importance of the thymus in immunity to P. berghei in rats (Brown, Allison & Taylor, 1968; Stechschulte, 1969), mice (Seitz, 1972; Clark & Allison, 1974) and hamsters (Wright, 1968; Chapman & Hanson, 1971), has been demonstrated and it is a reasonable assumption that this is also the case for P. chabaudi. In unpublished experiments we have found that anti-thymocyte serum extends the primary parasitaemia of P. chabaudi infected mice indicating an important role for T-cells. The cell transfer experiments described above set out to establish the role of T-cells in immunity to P. chabaudi and especially their possible role as helper cells in the production of protective antibody.

In many of the experiments the cell recipients were irradiated with 800 or 600 rads X-irradiation less than 24 hrs before cell transfer. It was hoped that this could reduce the contribution of the recipients own immune system to the control of the parasite. Both T- and B-cells are lost after irradiation, although a proportion of T-cells (Nossal & Pike, 1973), especially activated T-cells (Sprent et al., 1974), and B-cells (Pilarski & Cunningham, 1974) are relatively radioresistant. The responsiveness of irradiated animals to antigenic stimulation depends on the level of irradiation given, its timing with regard to antigen administration and the type of antigenic stimulation. In general, when antigen is given shortly after irradiation there is a severely depressed immune response (Taliaferro & Taliaferro, 1964) both cellular and humoral. In sub-lethally irradiated mice and lethally-irradiated mice reconstituted with stem cells there is a slow restoration of immunocompetence (e.g. response of mice to DNP-flagellin 30-80% restoration by 3-9 weeks, Nossal & Pike, 1973) which can be speeded up by the addition of T-cells (Lafleur, L et al., 1972). Macrophages are relatively radioresistant as far as some macrophage functions are concerned and sensitive with regard to others (Geiger & Gallily, 1974).

A feature of the experiments described in this report and the previous report was that irradiated mice frequently showed a non-specific resistance to P. chabaudi. The usual pattern was for the parasitaemia to become patent in the majority of irradiated and non-irradiated mice at about the same time but for the initial increase in parasitaemia to be slower in the irradiated mice. Cheers & Waller (1975) similarly found that 700 rad irradiated CBA mice were significantly more resistant to Brucella abortus and Listeria monocytogenes. Campbell et al. (1974) also reported non-specific resistance to Listeria in lethally irradiated CBA mice. Both groups of workers consider the non-specific resistance to be mediated through activated macrophages, possibly arising as a consequence of leakage of the gut flora into the blood vessels as a result of radiation damage (Slkivic, 1970). This latter worker examined carbon clearance rates in 600-1,000 rad irradiated mice and found that from about days 5-7 after irradiation the carbon clearance rate was elevated in the irradiated mice with a peak on day 14 and in some animals remained elevated for more than eight weeks: the larger the dose of irradiation the higher the carbon clearance rate. Alternatively the irradiation might inhibit or destroy suppressor T-cells (Dutton, 1972; Okumura & Tada, 1971), which allows, in some way, development of resistance to the parasites possibly through activated macrophages. Whatever the mechanism, however, the non-specific resistance in irradiated mice to P. chabaudi; it has to be taken into account when interpreting the activity of transferred lymphoid cells.

The first experiment demonstrated the need to inject small numbers of immune spleen cells intravenously if possible in order to confer a detectable immunity on the recipients. Filtration of immune spleen cells on a glass-wool column did not diminish the protective activity of the eluted spleen cells and in one of two experiments enhanced it. In both experiments the spleen cells were taken from mice which had almost certainly eradicated the infection, and the spleen was not significantly enlarged. The implication is that in these mice the effluent cells from the glass-wool column primarily lymphocytes were as important and probably more important in conferring protection on recipient mice than the macrophages retained on the glass-wool.

Above a limiting number of immune spleen cells, the degree of protection was directly related to the numbers of cells injected.

Nylon-wool columns were used to prepare enriched T-lymphocyte and B-lymphocyte populations. Although we have only just prepared mouse anti-theta and rabbit anti-thyomycyte sera to confirm that the effluent cells from the column are predominantly T-cells, the results of many other workers (Julius et al., 1973; Trizio & Ludkowicz, 1974) and our own observed changes in the proportion of cells with Ig receptors on them in the different populations leaves no doubt that the so-called enriched T-cell population is indeed so. Immunity could be transferred to irradiated and non-irradiated recipients (recent experimental data) with both glass-wool filtered immune spleen cells and enriched immune T-cells. The latter cells usually gave inferior protection although in recipients which had received only 600 rads irradiation or in non-irradiated recipients this was based on the appearance of patent relapse parasitaemias following regression of the primary parasitaemia to a subpatent level. The glass-wool filtered immune spleen cell populations usually contained approximately 25% more Ig bearing cells than the enriched T-cells population, indicating that these Ig receptor bearing cells play a measurable part in protecting the recipient mice, especially in heavily irradiated mice.

The enriched B-cell population, which contained about twice the number of Ig bearing cells as found in the glass-wool filtered cells, also gave protection but was less predictable in its efficiency. Usually the enriched B-cells gave protection as good as that with the enriched T-cells and in some experiments better protection in that recipients of the latter cells relapsed and the recipients of the former did not. It is very likely that the enriched B-cells population does contain some T-cells among the cells not bearing an Ig receptor.

The difference in the protection provided by enriched immune T-cells and glass-wool filtered cells, was best seen in 800 rad irradiated recipients suggesting that for the full expression of their protective activity they are partially dependent on radiosensitive cells in the recipients. One possibility is that for T-cell dependent anti-parasitic antibody production radiosensitive B-cells are necessary. Experiments in which T-cell recipients were also injected with bone-marrow cells from either immune or normal donors, or in which T-cell recipients also received enriched B-cell populations, did not consistently show that T-cells in the presence of these particular cells had greatly increased activity. Nevertheless removal of most of the B-cells from the glass-wool filtered immune cells frequently decreased their protective activity.

These experiments show therefore that immunity to P. chabaudi malaria in mice involves both T-cells and B-cells and phagocytic cells. The fact that in some experiments the enriched immune B-cell population gave better protection in the recipients than the enriched immune T-cells showed that the protection provided by the former population was not merely a consequence of the presence of the T-cells within it. In none of the experiments was there an immediate effect of the immune cells on the challenge inoculum, such as might be reflected in a delay in the onset of a patent parasitaemia. In the majority of experiments between 10^6 and 9×10^6 immune cells were transferred and the ratio of immune spleen cells to parasitized cell achieved in the recipients after challenge was usually 10:1 or less. This may have been too low for any immediately detectable cytotoxic activity by the immune cells. The fact that in some experiments the immune spleen cells came from donors last reinfected as long as 100 days before cell transfer (and at the time of cell transfer would no longer be infected), clearly demonstrated that in the immune T- (so called T_2 cells - Aranzo et al., 1975; Bach & Dardonne, 1973) and probably the B-cell population were long-lived memory cells and that these cell populations expanded on interaction with the parasite after challenge of the

As with the studies of other workers it is only speculation when we try to ascribe effector functions to T and B in malarial immunity. Coleman et al.(1975) examining immunity to P. berghei in mice describe cytotoxic spleen cells populations, including enriched T-cell populations, the cytotoxicity being amplified by immune serum. This particular study did not demonstrate the specificity of the cytotoxic effect which could have been directed against the red cell rather than the parasite. The in vitro proliferative response of sensitized lymphocytes to malarial antigen (Phillips et al., 1970; Kass et al., 1971; Wyler & Oppenheim, 1974) has been demonstrated. These observations, together with that of Coleman et al. (1976) that sensitized lymphocytes in the presence of malarial antigen can also liberate macrophage migration inhibition factor, show that a multitude of T-cell dependent activity including those initiated by lymphokines liberated by sensitized T-cells may play a part in protective immunity.

An important observation in the experiments described earlier was the occurrence of patent relapse parasitaemia in some immune cell recipients. Frequently the course of the patent primary parasitaemia, for example, in glass-wool filtered and enriched immune T-cell recipients (600 rad irradiated) followed the same course but in the T-cell recipients a relapse parasitaemia occurred. This relapse may be the result of antigenic variation by P. chabaudi and it may also be a consequence of regulatory mechanisms mediated through suppressor cells or the inhibitory activity of antibody or immune complexes.

Recommendations

There is undoubtedly a feeling among many malariologists at the present time that immunity to malaria parasites is not mediated solely through antibodies but that cellular mechanisms are implicated. Demonstration of, and observation of, these cellular mechanisms especially in human malaria are dependent on suitable in vitro culture procedures for these parasites. We believe that a microculture technique for P. falciparum, such as described in this report, is suitable for examination of both humoral and cellular immune mechanisms against this parasite. It is likely that because this parasite is readily maintained in vitro through at least $2\frac{1}{2}$ asexual cycles during which time gametocytes can differentiate, further attempts to improve the culture procedure, e.g. changing the medium, reducing the number of infected red cells in each culture and the addition of fresh red cells, will permit the parasite to be grown for a longer period. The ability of cryo-preserved P. falciparum to resume growth in vitro is of immense value. In immunity studies cryopreservation allows researchers to relate the immune response of an infected individual to a particular population of P. falciparum parasites which thereby takes account of the antigenic heterogeneity which undoubtedly exists in P. falciparum populations. On a wider front we see cryopreserved human red cells infected with P. falciparum being made available for such purposes as in vitro drug screening, metabolic studies, and experiments to improve culture procedures. Infected blood could be collected and deep-frozen in malarious areas and transported by air in liquid N_2 or dry ice to laboratories in, for example, the U.K. thereby reducing the need to plunder further the dwindling supply of exotic monkeys which provide P. falciparum infected red cells in the laboratory at the present time. Our recent investigations have shown that cryopreservation of infected red cells, following the procedure used by the West of Scotland Blood Transfusion Service, in which 17.5% glycerol is added to packed cells, gives a better red cell survival rate than the procedure described in this report. This method has similarities with

that described by Diggs et al., 1975.

It is recommended that the humoral and cellular response, e.g. lymphocyte cytotoxicity, to specific populations of P. falciparum from infected individuals be examined using cryopreserved parasites in micro-cultures as described in this report. It is likely that a simple in vitro procedure for measuring the level of protective immunity in individuals will prove to be of great value, for example, in the assessment of the susceptibility of a population to reinfection following a malaria control programme and in the longer term for determining the efficacy of an immunization programme.

The reason why malaria parasites persist in a semi-immune host may not simply be because of the parasite's ability to undergo antigenic variation. As discussed in Part II of the report it is suggested that the regulatory mechanisms responsible for controlling the various immunological responses to the malaria parasite, may determine whether, for example, those immune mechanisms which can lead to the parasite's eradication are expressed to the full. Antibodies, immune complexes and suppression T-cells are, for example, known to modulate the immune response to an antigen and all three could be considered as possible moderators of the protective immune response to malaria parasites. For example, Mackaness and colleagues (1974) found that the delayed response to sheep red cells in intact mice was non-existent but was readily detected in splenectomized mice. If eradication of malaria parasites is not solely the result of the activity of protective antibodies, as suggested by Phillips (1974), and that T-cell dependent cellular mechanisms are involved, then in splenectomized animals it could be expected that malaria infections would be terminated sooner than in non-splenectomized hosts.

It seems to the author that the whole area of the regulation and modulation of the immune response in relation to parasites in general and malaria parasites in particular is worthy of further investigation. Investigations involving the use of adjuvants as described in this report fall into this category and should continue because they, among other things, do present a means of artificially modulating the immune response to an antigen.

Our observation that the immunosuppressive effect of acute malaria and babesia infections extends to the immune response to concurrent infections of other parasites clearly is very relevant to the epidemiology and pathogenesis of many parasitic infections of man and his domestic stock. Although we have demonstrated the suppressive effect of malaria and trypanosome infections on the immune expulsion of one nematode infection of man, Trichinella spiralis, it is suggested that future studies concentrate on the interaction of these protozoa with parasites of man in the tropics where concurrent parasitic infections are common place. It is proposed in the first place to examine the interaction of malaria and trypanosomes and Schistosoma haematobium.

The results of the cell transfer studies, which should continue although delineating an important role for both T- and B-cells in immunity to malaria, have also revealed the complexity of the immune response to this parasite. The use of irradiated mice as cell recipients had aided these investigations but also raised some problems through the appearance of an initial increased resistance to the parasite in irradiated mice. The basis of this resistance is worth investigation because it could shed some light on these regulatory mechanism referred to above.

For example, it is known that T suppressor cells are radiosensitive and one possible explanation for the increased resistance after irradiation is that it is the result of the loss of these T suppressor cells.

In Technical Report ERO-5-74 I pointed out that a neglected area of research but one of great importance related to the immune response to the sexual forms of malaria parasites. I suggested that one way of interrupting transmission of the parasite would be to potentiate an immune response which decreased the appearance of, or decreased the infectivity of, gametocytes. There are strains of rodent malaria parasites available now which predictably show large numbers of gametocytes which could be used in such a study. In the previous Annual Report it was reported that in some of the in vitro cultures of P. falciparum gametocytes differentiated, in some cases developing from the merozoites which invaded red cells in vitro. It is suggested that those observations be followed up because not only could cultures provide a source of gametocytes for immunological studies but they could also provide information on the pattern of differentiation of gametocytes of P. falciparum.

Among malariologists there is at present a feeling of excitement and confidence that not only will continued progress be made towards the control of malaria but also on the way many biologically interesting phenomena will be discovered. I personally am very grateful to the U.S. Army that I have been able to make my own contribution, and communicate with and share this excitement with other research workers in various parts of the world.

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